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THE METABOLIC AVAILABILITY OF VITAMIN A (RETINOL) IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

BY

PRISCA JEBAIBAI TUITOEK ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR DEGREE OF
DOCTOR OF PHILOSOPHY

IN

NUTRITION

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ABSTRACT

Using non-diabetic control and streptozotocin-induced diabetic male Wistar rats of 4-6 week duration, the metabolic availability of vitamin A (retinol) was examined. Animals were fed ad libitum a nutritionally complete semi-synthetic diet either with (36 RE/g) or without (basal diet, 3.6 RE/g) vitamin A supplementation; or pair-fed the basal diet. Rats induced with diabetes developed hyperglycemia, increased urinary glucose and increased water intake within 48 hours. Despite the fact that the diabetic rats consumed nearly 50% more food than their corresponding controls, they gained less weight. These animals had decreased concentrations of vitamin A in the plasma and in the retina, while their total hepatic concentrations were markedly elevated. The circulatory vitamin A levels remained low while their hepatic concentrations were further elevated following supplementation of vitamin A, 10 times the amount in the basal diet. This reduced status of vitamin A in diabetic animals was not caused by its impaired intestinal absorption as evident by an in vitro study where the uptake of [^{3}H]retinol by the jejunum and ileum was not affected in the presence of diabetes. This was further supported by the fact that there was no difference in the hepatic concentrations of vitamin A in weight matched pair-fed control and diabetic animals. Free retinol concentrations in the liver were, however, higher in the diabetic than in control rats. These results suggest that there is an impaired mobilization of vitamin A from the liver to circulation.

Retinol is mobilized from its storage site in the liver and secreted into the plasma as a retinol-RBP complex (holo-RBP). The holo-RBP normally circulates in the plasma as a 1:1 molar complex with TTR. The concentrations of these carrier proteins were depressed in the plasma, liver and kidney of the diabetic animals; however, plasma albumin concentrations were unaffected. The decreased plasma vitamin A concentrations may be due to decreased synthesis or decreased stability (shorter half-life) of the carrier proteins.

Since zinc is an important factor for the hepatic synthesis of RBP and its metabolism is known to be affected in diabetes, the responses of circulatory vitamin A to zinc supplementation (120 ug/g diet) was examined. Further, the effect of insulin treatment (subcutaneous insulin pellet implant) on plasma and liver vitamin A concentrations was determined. The diabetes associated changes in vitamin A metabolism were reversed by insulin treatment but not by zinc supplementation.

The results of this study clearly point to the fact that STZ-induced diabetic rats are associated with reduced vitamin A status due to impaired availability of its carrier proteins. Although the mechanism leading to blindness in vitamin A deficiency and in diabetes is different, it is conceivable that retinopathy precipitated by diabetes can be further aggravated by vitamin A deficiency.

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LIST OF ABBREVIATIONS

ARAT	Acyl coenzyme A: retinol acyltransferase
BBM	Brush border membrane
CRABP	Cellular retinoic acid binding protein
CRALBP	Cellular retinaldehyde-binding protein
CRBP	Cellular retinol binding protein
CRBP(II)	Cellular retinol binding protein type two
IDDM	Insulin-dependent diabetes mellitus
IRBP	Inter-photoreceptor retinoid-binding protein
IU	International unit
LRAT	Lecithin: retinol acyltransferase
LDL	Low density lipoprotein
NIDDM	Non-insulin dependent diabetes mellitus
PEM	Protein energy malnutrition
RAR	Retinoic acid receptor
RE	Retinol equivalent
RBP	Retinol binding protein
RXR	Retinoid X receptor
TTR	Transthyretin

1. INTRODUCTION

Diabetes mellitus is a chronic disease characterized by abnormality in the metabolism of carbohydrate, lipid, and protein. It is the major cause of retinopathy and is currently a leading cause of new cases of legal blindness in adults (Palmberg, 1977; Moss et al., 1989). Vitamin A is known to play an essential role in vision; its deficiency will ultimately cause blindness (Oomen, 1974). There have been a few isolated reports suggesting that vitamin A metabolism may be of concern in diabetes mellitus. Lowered plasma levels of vitamin A in diabetic children were first reported by Mosenthal and Loughlin in 1944. A more recent study revealed that non-insulin dependent diabetic patients have increased plasma retinyl ester and lower retinol levels than normal subjects (Wako et al. 1986), suggesting an increased hepatic storage of vitamin A (Smith and Goodman, 1976). Other recent studies have shown that insulin-dependent diabetes mellitus patients have decreased concentrations of both plasma retinol and its carrier protein, retinol binding protein (RBP) when compared with non-diabetic subjects (Basu et al., 1989; Krempf et al., 1991; Martinoli et al., 1993). Since cutaneous signs of carotenemia and elevated serum β-carotene (a precursor of vitamin A) levels have been found in patients with diabetes mellitus, it has been suggested that the diabetic state interferes with the conversion of β-carotene to vitamin A (Gouterman and Sibrack, 1980). Moreover, zinc is known to be an important factor for the hepatic synthesis of RBP (Smith, 1982) and its metabolism is perturbed in the presence of diabetes mellitus (Kinlaw et al., 1983; Lau and Failla, 1984; Pai and Prasad, 1988; Uri-Hare et al., 1992).

Despite these observations, the metabolism of vitamin A in diabetes has not been adequately studied. The objective of this thesis was to further elucidate the reasons for reduced circulatory vitamin A in diabetes. A good model to carry out this study was identified to be streptozotocin-induced diabetic Wistar rat. The effects of supplementation of vitamin A and zinc; pair-feeding; and the administration of insulin on the status of vitamin A in diabetic rat were determined. The effect of diabetes on vitamin A carrier proteins and the absorption of vitamin A were also studied.

DIABETES MELLITUS

Diabetes afflicts a large number of people of all social conditions worldwide. It is estimated that the incidence of insulin-dependent diabetes mellitus (IDDM) varies from 0.6 per 100,000 in Korea and Mexico to 35.3 per 100,000 in Finland (Karvonen et al., 1993). Non-insulin dependent diabetes mellitus (NIDDM) is a common disorder with a prevalence of 3-6% in adults in most western populations and up to 25% among some groups such as the American Indians (Galloway, 1990). However, prevalence of diabetes still remains largely unknown in certain parts of the world such as in Africa and Asia because data are sparse.

The metabolic abnormalities of diabetes mellitus result primarily from a decrease in insulin secretion and diminished insulin activity in target tissues (Unger and Foster, 1992). It is classified into two major categories: IDDM or type I diabetes and NIDDM or type II diabetes. IDDM results primarily from lack of insulin due to profound autoimmune β-cell destruction and is characterized by hyperglycemia and ketoacidosis (Olefsky, 1985). NIDDM is a multifactorial disorder which results from decreased insulin secretion and insulin resistance and is characterized by fasting hyperglycemia and an excessive postprandial rise in the basal plasma glucose concentration (DeFronzo et al., 1989). Approximately 90% of all diabetic patients are affected by NIDDM, of whom 80% are overweight.

Maintenance of the plasma glucose concentration is critical to human survival, because it is the predominant metabolic fuel utilized by the central nervous system under most conditions (Cryer, 1992). The central nervous system has no mechanism to store glucose. Hence, brief shortage of glucose can cause profound brain dysfunction and if prolonged may cause brain death. Improved control of the diabetic state (i.e., maintenance of nearly normal levels of blood glucose concentrations) may reduce the rates of occurrence of some complications commonly associated with diabetes (Benjamin and Sacks, 1994). Progressive damage to the eyes (retinopathy), kidney (nephropathy), nerves (neuropathy) and arteries (cardiovascular disease) represent the major threat to the health and life of diabetic patients.

Insulin is secreted by the pancreatic β-cells; it is an anabolic hormone which acts in different organs and tissues in varied ways. Insulin acts by binding to specific insulin receptors which are transmembrane glycoproteins and present in all insulin target tissues (Kahn and White, 1988). Insulin is required for the uptake and utilization of glucose by insulin-dependent tissues such as the skeletal muscle and adipose tissue. It promotes the synthesis of glycogen, protein and lipid, as well as amino acid uptake and glucose oxidation (Cryer, 1992). It inhibits glycogenolysis, ketogenesis, gluconeogenesis and lipolysis. In the absence of insulin the hormones, such as glucagon, catecholamines, growth hormone and cortisol, favoring catabolism of protein and lipids, and the raising of blood glucose, operate without opposition.

VITAMIN A AND DIABETES

Diabetes accounts for approximately 5,000 new cases of blindness in the U.S.A. each year, and blindness is 25 times more common in the diabetic than in the non-diabetic population (Ferris, 1993). It is estimated that between 50-90% of diabetic individuals will develop some form of retinopathy in a 20 year period of diagnosis. The early stage of retinal involvement by diabetes is nonproliferative retinopathy which is characterized by such changes as microaneurysms, dot hemorrhages, exudates, and retinal edema. In this stage, the retinal capillaries leak proteins, lipids or red cells into the retina. Proliferative retinopathy occurs later and involves the growth of new capillaries and fibrous tissue within the retinal and into the vitreous humour.

An early sign of vitamin A deficiency in the eye is night blindness in which one's ability to see in dim light is impaired. Later, ocular lesions such as conjuctival xerosis (dryness), Bitot's spots, keratomalacia and xerophthalmia may occur. Vitamin A deficiency blindness is endemic in many developing countries and is mainly a consequence of poor nutrition. It is estimated that 5-18 million children, mostly from the developing countries are subclinically deficient in vitamin A to a level that increases their risk of mortality and perhaps morbidity (UNICEF, 1990). In developed countries however, the prevalence of vitamin A deficiency is low and occurs secondary to certain diseases such as cystic fibrosis, severe intestinal and liver diseases, and severe defects in lipid absorption (Gibson, 1990). The retinal pigment epithelial cell is very rich in vitamin A and contains approximately 87% of the total vitamin A in the human eye (Bridges, 1984). One of the key functions of this cell is to provide rods with a supply of 11-cis retinal, an aldehyde of vitamin A, which is the key to the formation of rhodopsin, the visual pigments. Rhodopsin is generated when the protein opsin in the rods of the retina combines with 11-cis retinal. The complex is split in response to light, yielding opsin and all-trans retinal and generating the visual-response signal.

Although the underlying mechanism for blindness is different in vitamin A deficiency and in diabetes, it is conceivable that retinopathy, precipitated by diabetes, can further be aggravated in the presence of a vitamin A deficiency. It is therefore important that the metabolism of vitamin A is further studied in order to obtain a better understanding of the relationship between diabetes and vitamin A metabolism.

There are a few reports suggesting that vitamin A metabolism may be of concern in diabetes mellitus. A greater increase in plasma retinyl ester and lower plasma retinol levels have been observed in patients with NIDDM than in normal subjects (Wako et al., 1986). Vitamin A is normally transported in the plasma as retinol bound to retinol-binding protein (RBP). However, in hypervitaminosis, increased plasma retinyl esters have been shown to be associated with lipoproteins, suggesting an increased hepatic storage of vitamin A (Mallia et al., 1975; Smith and Goodman, 1976). The liver is capable of storing large amounts of vitamin A, but following excessive intakes, it becomes saturated with the vitamin so that considerable amounts of retinyl ester spill over into the circulation. Vitamin A which is not bound to RBP may cause nonspecific toxicity to cell membranes. However another study found that there was an increase in plasma retinol levels in NIDDM patients when compared to non-diabetic control subjects (Krempf et al., 1991). The reason for the difference in these two studies is uncertain but different metabolic controls in the patients studied may be a likely explanation since subjects in the first study ranged in age between 29 and 87 years old. In the latter study, patients were hyperinsulinemic and it is possible that high amounts of vitamin A from the liver were mobilized to the plasma as this has been shown to occur in insulin-treated rats (Bowles, 1967).

In IDDM however, studies consistently show that patients have decreased concentrations of both plasma retinol and its carrier protein, RBP when compared with non-diabetic subjects (Basu et al., 1989; Krempf et al., 1991; Martinoli et al., 1993). Unlike retinol, plasma concentrations of α-tocopherol, another fat soluble vitamin, were not significantly reduced in diabetic patients. In these studies, no significant correlations were found between glycosylated hemoglobin and plasma vitamin A concentrations. In STZ-induced diabetic rats, plasma vitamin A concentrations were significantly lower at 1, 3 and 6 weeks after the onset of diabetes than in the corresponding controls (Basu et al., 1990). In contrast, the liver vitamin A concentrations were significantly elevated in the diabetic groups at week 3 and 6 when compared to controls. This increase in liver concentrations of vitamin A was attributed in part to increased (> 40%) food intake by the diabetic animals, since hepatic vitamin A is proportional to its intake (Hicks et al., 1984). The decreased plasma levels and increased hepatic storage of vitamin A however, suggest that vitamin A mobilization from the liver may be impaired in diabetes. It has been demonstrated in rats that retention of vitamin A in the liver could be influenced by insulin, which caused a reduction in the liver content of vitamin A (Bowles, 1967). Thus an insulin deficiency could impair the release of vitamin A from the liver and therefore be responsible for the low plasma retinol concentrations observed in diabetics. Vitamin A is mobilized from the hepatic stores as the unesterified free retinol and bound to its carrier, RBP. It is also possible that the diabetic state leads to decreased rates of RBP synthesis and probably secretion by the liver, which is in turn may be responsible for the lower levels of vitamin A and RBP in plasma. It is further possible that decreased plasma RBP levels are due to increased renal excretion of RBP as this has been observed in IDDM patients (Rowe et al., 1987; Holm et al., 1987). In normal states, small proteins that are filtered with relative ease through the glomeruli are taken up and catabolized in the tubular cells (Mogielnicki et al., 1971). In tubular dysfunction when the glomerular filtration rate is normal, protein filtration continues unchanged, but tubular uptake and catabolism decrease, hence more protein appears in the urine.

Elevated levels of β-carotene have been observed in patients with diabetes (Cohen, 1958; Kondirci et al., 1993). This has been attributed, in part, to increased intake of vegetables containing β-carotene (Cohen, 1958). However, it has also been suggested that impaired conversion of β-carotene to vitamin A may occur, especially in hyperlipemic states (Gouterman and Sibrack, 1980).

Zinc is known to be important for the hepatic synthesis of RBP (Smith, 1982) and evidence shows that the development of diabetes mellitus may lead to zinc deficiency (Kinlaw et al., 1983; Lau and Failla, 1984; Pai and Prasad, 1988; Sjogren et al., 1988; Uri-Hare et al, 1992). A study involving 20 NIDDM patients, revealed that 25% had depressed serum zinc concentrations and 100% had hyperzincuria (Kinlaw et al., 1983). Patients with IDDM and NIDDM have hyperzincuria as well as decreased levels of zinc in the serum, lympocytes, granulocytes, and platelets (Pai and Prasad, 1988), suggesting a zinc deficiency. In 30 newly diagnosed IDDM children aged between 3-15 years, serum zinc concentrations were lower at 6 months of diagnosis but were normalized to control levels after two years of insulin treatment (Kobbar et al., 1988). In STZ induced diabetic rats, zinc excretion was found to be 3.4-fold more than in controls on the 14th day (Lau and Failla, 1984), and this was significantly reduced when treated with insulin, suggesting that altered hormonal status was the primary cause of increased urinary zinc loss. It has therefore been suggested that abnormal zinc metabolism could play a role in vitamin A metabolism in diabetes mellitus. Zinc is also involved in the structural form of storage of insulin as complexes of zinc and insulin in varying ratios are stored in the pancreatic cells (Emdin et al., 1980). Furthermore, zinc chelation seems important to the mechanism of action of the chemical diabetogenic agents such as streptozotocin (Epand et al., 1985). This is supported by the fact that induction of chemical diabetes is accompanied by a loss of zinc from the pancreatic β-cells.

The importance of zinc in human health is well established. Zinc is required for the activity of over seventy enzymes and is considered important for cell division, DNA and protein synthesis. Growth and development, testicular function, taste acuity, dark adaptation, wound healing and cell mediated immunity are known to be impaired as a result of zinc deficiecy in humans. Impaired dark adaptation in humans possibly results when metabolizable zinc is inadequate to maintain the activity of retinene reductase, a zinc-metallo alcohol dehydrogenase of the retina, which is involved in the conversion of retinol to retinal (Smith, 1982). Age-related macular degeneration, a leading cause of vision loss among the elderly, is known to be due to a reduction in availability of zinc in the retinal pigment epithelium (Anonymous, 1990).

The underlying mechanism for the subnormal plasma vitamin A and its increased hepatic concentration in diabetics (Basu et al., 1990) is not properly understood. The increased hepatic store of vitamin A has been attributed, at least in part, to an increased food intake by the diabetic animals. It is possible that there might be an increased absorption of vitamin A in diabetes because of its lipid solubility. Absorption of lipids such as fatty acids and cholesterol is increased in experimentally-induced diabetic rats (Thomson, 1980). This increase in the absorption of lipids has been attributed to a change in the dimensions and characteristics of the major intestinal barriers to intestinal absorption, the unstirred water layer and the permeability properties of the microvillus membrane. In diabetic rats, there is a decrease in the effective resistance of the unstirred water layer and an increase in the incremental change in free energy associated with the uptake of fatty acids in comparison with control rats (Thomson, 1983).

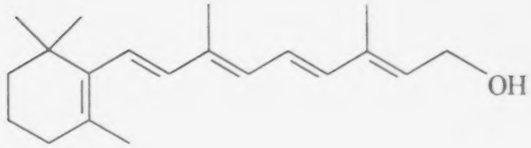
The nutritional status of vitamin A, together with the factors affecting its metabolism in diabetes, have been examined only in few studies. Thus there is need for further study of vitamin A metabolism in diabetes.

OVERVIEW OF VITAMIN A METABOLISM

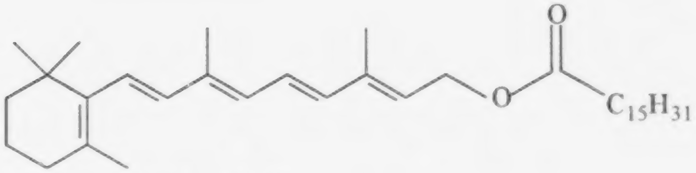
The term "vitamin A" refers to all compounds that exhibit the biological activity of retinol, whereas "retinoids" include the natural forms of vitamin A as well as the many synthetic analogues of retinol, whether or not they have biological activitiy (Blomhoff et al., 1992). Vitamin A occurs physiologically as the alcohol (retinol), the aldehyde (retinal), the acid (retinoic acid), and the ester (retinyl ester) (Figure 1-1). Vitamin A has a number of functions in the body. It is necessary for vision, bone development, reproduction and normal differentiation of epithelial tissues. These functions are mediated by different forms of the molecule. All-trans retinol is oxidized to retinal, which is involved in the visual cycle (Wald, 1968). This is a reversible reaction, as retinal can also be converted to retinol. Oxidation of retinol also produces irriversibly retinoic acid which is capable of promoting growth and differentiation of epithelial tissues, but unlike retinol, does not support visual and reproductive functions (Dowling, 1960) and reproduction (Thompson et al., 1964). Thus vitamin A deficient animals supplemented with retinoic acid will grow normally but will be blind.

Intake and absorption

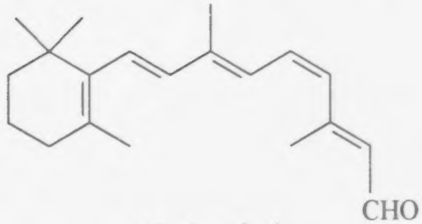
Vitamin A is consumed mainly as carotenoids (provitamin) from plant sources and as retinyl esters (preformed vitamin) from animal products. Vitamin A compounds are all fat soluble; thus normal fat digestion and absorption are necessary for their absorption (Goodman and Blaner, 1984). The common dietary



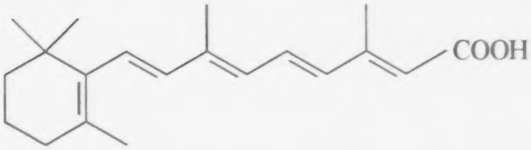
all-trans retinol



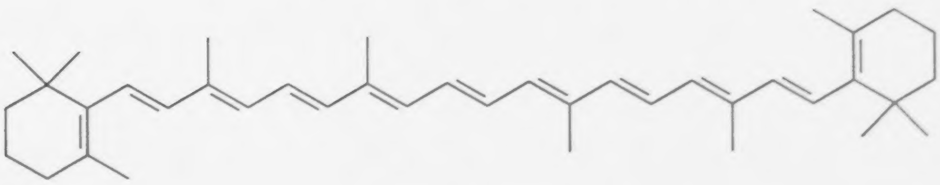
retinyl palmitate



11-cis retinal



all-trans retinoic acid



β-carotene

FIGURE 1-1: Structural formulae of some biologically active and naturally occurring forms of vitamin A.

sources of vitamin A are various dairy products, such as milk, cheese, and butter, eggs, liver and fish oil, mainly as retinyl palmitate, whereas as β-carotene sources include fruits, green leafy and yellow vegetables such as carrots, pawpaws and mangoes.

After foods are ingested, preformed vitamin A of animal tissues and the provitamin carotenoids of vegetables and fruits are released from proteins by the action of pepsin in the stomach and proteolytic enzymes in the small intestine (Olson, 1991). In the stomach the free carotenoids and retinyl esters tend to congregate in fatty globules, which then enter the duodenum. In the presence of bile salts, the globules are broken up into smaller lipid congregates, which can be more easily digested by intestinal enzymes (Olson, 1991). The resultant mixed micelles, which contain retinol, carotenoids, sterols some phospholipids, mono- and diglycerides and fatty acids, help to transport these lipids through the aqueous diffusion barrier (the unstirred water layer) to the epithelial cell membranes. The bioavailability and the digestion of vitamin A and carotenoids are affected by the overall nutritional status of the individual and the integrity of the intestinal mucosa. Nutritional factors of importance are proteins, fat and vitamin E (Olson, 1991). For example, protein is important for the synthesis and release of digestive enzymes; while fat in the diet markedly stimulates absorption of vitamin A by enhancing bile acid production and by providing a vehicle for its transport and absorption; and vitamin E protects carotenoids and retinol from oxidation during the digestive processes. Bile salts, which are detergents, promote the rapid cleavage of retinyl and carotenoid esters and assist in the transfer of these lipids into mucosal cells.

Absorption of carotenoids (predominantly β-carotene) is by passive diffusion and up to 50% of dietary intake is absorbed by humans (Blomhoff et al., 1991). It is assumed that 12 μg of mixed carotenoids in a typical diet, or 6 μg of β-carotene, is equivalent to 1 μg of retinol or 1 μg retinol equivalents (Olson, 1994). The reason for these differences is due to reduced absorption of carotenoids and conversion to retinol (Olson, 1994). The conversion of β-carotene to retinol in the enterocytes involves the production of two retinal units by the enzyme β-carotene-15,15'-dioxygenase and reduction to retinol by retinal reductase (Goodman and Blaner, 1984). In humans and some other species, a significant proportion of carotenoids are absorbed intact and transported from the gut via the intestinal lymph. While retinol is absorbed readily in solubilized form, carotenoids have an absolute requirement for bile salts (Olson, 1991).

Retinyl esters from the diet are hydrolyzed in the intestine before absorption into the enterocytes. Pancreatic lipase has been assumed to be responsible for the hydrolysis of the major diet retinyl ester (palmitate). However, recent reports in rats and humans show that retinyl esters are hydrolyzed by two enzyme systems located on the brushborder membrane (BBM); a pancreatic origin lipase (also called cholesterol ester hydrolase) which is responsible for hydrolysis of short-chain retinyl esters (mainly C6 and C8) and an enzyme intrinsic to the BBM which hydrolyses long-chain retinyl esters (mainly C12, C14 and C16; highest activity, C16), in the absence of pancreatic secretions (Rigtrup and Ong, 1992; Rigtrup et al., 1994). Retinol in physiological concentrations is apparently absorbed by facilitated diffusion (i.e. a protein carrier mediated process), whereas, at pharmacological levels it is absorbed by passive diffusion (Hollander, 1981; Said et al., 1988). It is estimated that the overall absorption efficiency of dietary vitamin A is less than 75% from animal foods and it depends on both the quality and quantity of dietary fat (Norum and Blomhoff, 1992). Figure1-2 shows the major pathways of vitamin A metabolism.

Transport and storage

Retinol absorbed into the enterocytes is reesterified to retinyl esters before accumulation into chylomicrons for transport via the lymphatic system to the liver. There are two enzymes which have been identified as being important for the esterification of retinol in the enterocytes. The acyl coenzyme A: retinol acyltransferase (ARAT) (Helgerud et al., 1982, 1983; Rasmussen et al., 1984) and lecithin: retinol acyltransferase (LRAT) (Ong et al., 1987; MacDonald and Ong, 1988). It has been found that LRAT esterifies retinol that is complexed with cellular retinol binding protein type two (CRBP(II)) (MacDonald and Ong, 1988) and uses phosphatidylcholine as the endogenous acyl donor, as a source of fatty acid for esterification of retinol. In contrast, uncomplexed retinol found in membranes may be esterified by ARAT. Thus, it has been suggested that LRAT esterifies retinol during absorption of a "normal" load of retinol, and ARAT esterifies excess retinol when large doses are absorbed and CRBP (II) becomes saturated (Blomhoff et al., 1992).

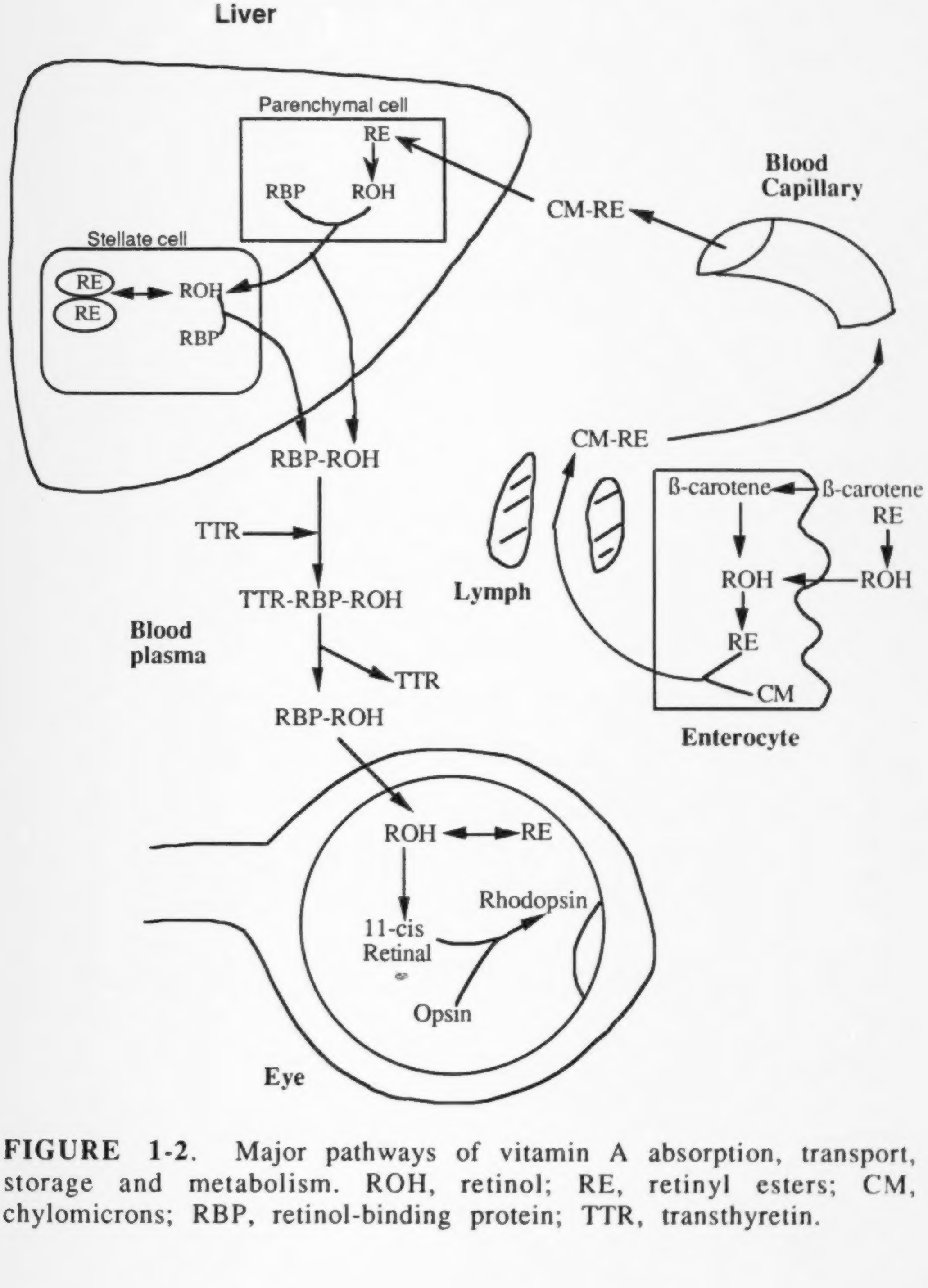

Liver
Parenchymal cell
RE
RBP
ROH
Stellate cell
RE
RE
ROH
RBP
CM-RE
Blood Capillary
RBP-ROH
TTR
TTR-RBP-ROH
Blood plasma
TTR
RBP-ROH
CM-RE
ß-carotene
ß-carotene
RE
ROH
ROH
RE
CM
Lymph
Enterocyte
ROH
RE
Rhodopsin
11-cis Retinal
Opsin
Eye

FIGURE 1-2. Major pathways of vitamin A absorption, transport, storage and metabolism. ROH, retinol; RE, retinyl esters; CM, chylomicrons; RBP, retinol-binding protein; TTR, transthyretin.

In the absorptive cell, retinyl esters, lipids and fat-soluble vitamins and in certain species such as humans, some carotenoids are packaged together in the endoplasmic reticulum into chylomicrons. Chylomicrons are then processed through the Golgi complex and transported via the thoracic duct lymph into the general circulation. In circulation, chylomicrons undergo various processes such as transfer of various lipids to other lipoproteins or to cell membranes resulting in chylomicron remnants (Blomhoff et al., 1991). Chylomicron remnants are taken up by the liver via the low density lipoprotein (LDL) receptor and probably the LDL receptor-related protein endocytosis. Most of the chylomicron retinyl esters are taken up by the liver (Goodman et al., 1965). It was shown that when retinyl ester-labeled chylomicrons were injected into rats, measurable amounts of radioactivity were recovered in adipose tissue (3-10%), skeletal muscle (4-7%), kidneys (0.4-4%), and the rest of carcass (0-10%) compared with liver (70%), at times up to 3 hours. These findings were supported by later studies which showed that some chylomicrons are also delivered to extrahepatic tissues such as muscle, adipose tissues, kidneys (Blomhoff et al., 1982) and bone marrow (Hussain et al., 1989). Vitamin A is stored in the liver as retinyl esters, and the liver is the primary site of whole body vitamin A storage. It is estimated that more than 90% of the whole body vitamin A is stored in the liver of vitamin A-sufficient animals with plasma content representing only about 1% of the total body reserves of this vitamin (Ostrowski et al., 1989).

Once the retinyl esters have been taken up by the liver parenchymal cells (hepatocytes), rapid hydrolysis takes place, catalyzed by retinyl ester hydrolase (Harrison and Gad, 1989). The resulting retinol binds to RBP, presumably in the endoplasmic reticulum, and RBP-retinol complex is then transfered to the Golgi compartment for secretion (Blaner et al., 1989). It is either secreted into circulation or transfered to stellate cells where it is stored or probably secreted into circulation (Blomhoff et al., 1988). It was shown that in chylomicrons containing [^{3}H]retinyl palmitate or retinyl [^{3}H]palmitate injected intravenously into rats, the labeled retinol and the labeled palmitate moieties were initially taken by the parenchymal cell. However, after some time, only the radioactive retinol could be found in the stellate cells, while the labeled palmitate was retained in the parenchymal cells. About 90% of the hepatic vitamin A is present in the stellate cells mainly stored as retinyl esters (Blomhoff et al., 1988; Trøen et al., 1994). Esterification of retinol in the stellete cells appears to involve two enzymes similar to those found in the enterocytes; ARAT and LRAT, with the participation of cellular RBP type one (CRBP(I)) (Ong et al., 1988; Yost et al., 1988). The secretion of RBP-retinol complex from the liver to plasma is influenced by vitamin A status. In severe vitamin A deficiency, secretion of RBP-retinol is reduced such that plasma concentrations of RBP and retinol are decreased, but vitamin A repletion leads to an immediate increase in the secretion of RBP-retinol from the liver (Smith and Goodman, 1979; Blaner et al., 1989).

Vitamin A mobilization from the liver stores to the plasma and delivery to the peripheral tissues is controlled by a regulated system that ensures constant plasma concentrations inspite of normal fluctuations in daily intake. This involves the secretion of retinol combined with RBP (21 Kilodaltons) into the plasma where it reversibly complexes with another protein, transthyretin (55 Kilodaltons) to form a 1:1 molar ratio. The binding of RBP to TTR stabilizes the association of retinol with RBP and also prevents glomerular filtration and renal catabolism of RBP (Goodman, 1984). When RBP is bound to TTR, its half life is approximately 12 hours, whereas free RBP is removed much more rapidly (half life, about 4 hours) (Vahlquist et al., 1973). It is estimated that approximately 95.5% of plasma retinol is present as TTR-RBP-retinol, 4.4% as RBP-retinol, and 0.14% as unbound retinol (Blomhoff et al., 1991).

In prolonged excess intake of vitamin A, up to 65% of plasma vitamin A is transported in the form of retinyl ester compared to less than 5% found in a normal healthy person (Smith and Goodman, 1976). During excess vitamin A intake, it is thought that the hepatic storage capacity of vitamin A exceeds its limits such that retinyl esters accumulate in the lipoproteins which are secreted into the plasma. Vitamin A carried in the lipoproteins is apparently available to the cell membranes and has been attributed to toxicity and damaging of these membranes during hypervitaminosis A (Mallia et al., 1975; Goodman, 1984).

The circulatory retinol bound to RBP and entering the choriocapillaries of the eye is taken in by membrane receptors on the basolateral surface of the RPE (Bok, 1990). In the cell, all-trans retinol is then bound by CRBP, delivered to membrane-bound retinyl ester synthetase (probably synonymous with LRAT). This enzyme removes a fatty acid, normally palmitic, from phosphotadyl choline and transfers it to retinol to form retinyl esters which is then stored or acted on by isomerase enzyme to form 11-cis retinol. The 11-cis retinol is then oxidized to 11-cis retinal, which diffuses to the interphotoreceptor matrix and then to the rod outer segments for rhodopsin biosynthesis. When light bleaches the rhodopsin, 11-cis retinal is converted to all-trans retinal which is then reduced to all-trans retinol. All-trans retinol diffuses back to the interphotoreceptor matrix and to the RPE for esterification and reisomerization.

The mechanism responsible for retinol uptake from plasma into target tissues is not fully understood. It has been suggested that retinol may partition into plasma membranes (Creek et al., 1993) or may enter cells as a result of fluid-phase endocytosis without the use of a cell surface receptor (Blomhoff et al., 1991). However, receptor-mediated uptake of retinol appears to be the most important mechanism (Heller and Bok, 1976; Blomhoff et al., 1991; Bavik et al., 1992). When RBP is taken up by specific cells, the binding of labeled RBP is blocked by excess unlabeled RBP and uptake in vivo shows a specific tissue distribution. Transthyretin seems to inhibit the binding of RBP, indicating that retinol-RBP (rather than retinol-RBP-TTR) is the vehicle that delivers retinol to the receptor (Blomhoff et al., 1991). Retinol binding protein receptors have been identified in retinal pigment epithelial cells (RPE), hepatocytes, stellete cells, testis, brain barriers, placental brush border membranes and keratinocytes.

Once retinol enters a target cell, it is bound by the cellular retinol-binding proteins (CRBP) which are widely distributed among tissues. The proteins may not only protect retinol from oxidation within the cell cystol, but also may serve as a carrier to its intracellular site of action (Olson, 1991). CRBP (I) differs from plasma RBP in that its molecular weight is lower, 15,700 instead of 21,000 and the two proteins do not immunologically cross-react (Olson, 1991). Many specific intracellular retinol- and retinoic acid-binding proteins (CRBPs and CRABPs, respectively) have been identified. It appears that CRBP (I) is most abundant in such tissues as the liver, lung, kidneys, epidydymis and testes whereas CRABP (I) is most concentrated in the testis, skin and eyes (Blomhoff et al., 1991). The CRBP (II) is much more restricted to intestinal absorptive cells where it is important in the esterification of retinol by LRAT (MacDonald and Ong, 1988). Two binding proteins are found solely in the eye (Bok, 1990). Cellular retinaldehyde-binding protein (CRALBP), which is found in the Müller cells of the retina and in the RPE, but not in the rod outer segments binds both 11-cis retinal and 11-cis retinol. An inter-photoreceptor retinoid-binding protein (IRBP) has been identified in the extracellular space between the retinal pigment epithelial cells and the photoreceptor cells which binds all-trans and cis retinol.

Another group of nuclear receptors which binds retinoic acid has recently been discovered (Wolf, 1993). Retinoic acid receptors (RARα,β,γ) bind to retinoic acid to interact with responsive elements in various genes involved in development and differentiation. Additionally, there exists a class of structurally distinct nuclear receptors referred to as retinoid X receptors (RXRα,β,γ) which bind 9-cis-retinoic acid as ligand and may function in regulation of genes involved in intermediary metabolism (Wolf, 1993).

Excretion

Approximately 5-20% of ingested vitamin A and a larger percentage of carotenoids are not absorbed in the intestinal tract and are excreted in the feces (Olson, 1994). A significant portion (10-40%) of the absorbed vitamin A is oxidized and/or conjugated in the liver and then secreted into the bile (Olson, 1994). Oxidation of the biologically active all-trans retinol to retinal involves the nonspecific enzyme, alcohol dehydrogenase and the more specific retinol dehydrogenases (retinal reductases) which are present in many tissues including the eye, liver and intestine. In the eye, the reaction provides retinal for the rhodopsin regeneration. In the liver, further oxidation of retinal is irreversible and produces retinoic acid, which is rapidly metabolized to more polar metabolites. Most oxidized products are either excreted in the urine or conjugated in the liver with glucuronic acid and taurine, the principal metabolites being retinyl β−glucuronide, retinoyl β−glucuronide and retinotaurine for excretions into the bile. Some of the biliary metabolites are absorbed in the intestine and transported back to the liver while the rest of the biliary metabolites are excreted in the feces. In quantitative terms, of the dietary intake of vitamin A, an average of 10% is not absorbed, 20% appears in the feces via bile, 17% is excreted in the urine, 3% is excreted as carbon dioxide and 50% is stored, primarily in the liver (Olson, 1994).

Factors affecting plasma vitamin A and RBP metabolism

Vitamin A status

Plasma retinol levels are homeostatically regulated so that they are maintained within a narrow range despite wide variations in dietary vitamin A intake, except in a state of severe deficiency. In vitamin A deficiency, hepatic stores are gradually depleted in order to maintain adequate plasma retinol with its levels falling when hepatic concentrations are below 20 μg/g liver (Olson, 1984). The release of retinol from the liver requires binding with RBP.

The secretion of RBP from the hepatocyte is strictly regulated by availability of its ligand, retinol. In vitamin A deficiency, the secretion of RBP from the hepatocyte is specifically inhibited so that the plasma level falls and apo-RBP accumulates in the liver (Muto et al., 1972). RBP is apparently synthesized in the endoplasmic reticulum, secreted to the Golgi complex and when vitamin A deficient rats are repleted with retinol, RBP is rapidly secreted into the plasma. The retinol-induced secretion of RBP has been shown to be independent of protein synthesis (Soprano et al., 1982). This is because during deficiency of vitamin A, the RBP concentration in the liver is considerably increased and when vitamin A is repleted, the liver concentration of RBP decreases while its plasma concentration increases.

Vitamin A depletion in rats was found to decrease both serum retinol and RBP levels after 25 to 30 days (Muto et al., 1972). However, hepatic RBP of the deficient rats were about 4 times more than in control rats. When vitamin A was given orally, a very rapid increase in serum RBP values were observed. In another study (Smith et al., 1973), where vitamin A deficient rats were injected with lymph chylomicra containing graded amounts of vitamin A (mainly as retinyl esters), a rapid increase in the serum values of RBP were observed, with the magnitude of response directly dose-related to amounts of vitamin A injected. When protein synthesis inhibitors were given to another group of vitamin A deficient rats, injections of vitamin A increased serum RBP, suggesting that hepatic secretion and not synthesis of RBP was defective. Colchicin (an inhibitor of plasma protein secretion) markedly inhibited the vitamin A-stimulated secretion of RBP from the liver (Smith et al., 1980). The block in RBP secretion from the liver was found to be specific to RBP because neither vitamin A deprivation nor repletion of vitamin A-deficient rats significantly affected plasma TTR levels (Navab et al., 1977).

Protein status (PEM)

Vitamin A absorption, transport and metabolism are dependent upon several enzymes and specific proteins synthesized in the body.

Thus protein becomes an important factor in the maintenance of vitamin A status. In protein-energy malnutrition (PEM), plasma RBP, TTR and vitamin A are reduced due to a defective hepatic synthesis of the proteins for mobilization of vitamin A or to an inadequate intake of vitamin A (Goodman, 1984). Since both RBP and TTR are rapidly turning over plasma proteins (i.e., with short half lives), it can be anticipated that their plasma levels might provide a sensitive index for mild degrees of PEM as compared to plasma levels of more slowly turning over proteins such as albumin (Goodman, 1984). Furthermore, RBP contains high proportions of aromatic and essential amino acids. Thus protein deficiency or regular intake of incomplete proteins exert pronounced effects on RBP synthesis (Ganguly, 1989).

Several earlier studies of children with kwashiorkor showed that their serum levels of retinol, RBP, TTR, albumin and total protein were lower than in normal children (Arroyave et al., 1961; Smith et al., 1973; Venkatswamy et al., 1977). However, liver biopsy specimens revealed sufficient hepatic storage of vitamin A (Arroyave et al., 1961). When incomplete proteins and sufficient energy were given to the children with kwashiorkor, without any vitamin A supplementation, 50% of the children's plasma retinol, RBP and TTR became normal (Smith et al., 1973). In another study, it was observed that the more xerophthalmic the children were, the lower the serum RBP and TTR levels (Venkatswamy et al., 1977). Administration of vitamin A alone to these children did not restore the plasma levels but normal levels were achieved when a high-protein diet containing β-carotene and vitamin A was given.

Serum albumin level has generally been used as measure of protein status. However, the long half-life (14 days) of serum albumin limits its usefulness in assessing nutritional status. Hence recent studies suggest RBP and TTR are more sensitive indicators for assessing early PEM (Shetty et al. 1979; Wade et al. 1988; Polberger et al., 1990), because of their relatively short half-lives (12 hours and 2-3 days, respectively). In obese women where albumin and transferrin failed to respond to short-term nutritional changes, such as restriction of either protein or energy intake, plasma TTR and RBP levels were decreased (Shetty et al., 1979). Moreover, protein rather than total energy intake seems to provide the main contribution to the changes found in plasma RBP and TTR in preterm infants (Polberger et al., 1990).

Zinc status

Results of several studies have shown that zinc plays an important role in the maintenance of vitamin A concentration in the blood (Smith, 1982). In conditions manifested by low zinc status in humans, such as alcoholic cirrhosis (Smith and Goodman, 1971; McClain et al., 1979), PEM (Shingwekar et al., 1979) and preterm infants (Hustead et al., 1988), zinc supplementation improved vitamin A status. Zinc-deficient rats (Brown et al. 1976), swine (Stephenson and Earle, 1956) and lambs (Sarawat and Arora, 1972) have shown low plasma vitamin A levels despite adequate vitamin A concentrations in the liver. Zinc supplementation restored the level of plasma vitamin A to normal. It has also been demonstrated that a single intraperitoneal injection of large doses of zinc causes an abrupt rise in plasma retinol and a decline in hepatic retinol as compared to saline-injected control rats (Ette et al., 1979), suggesting an increase in vitamin A from the liver. Smith et al. (1974) have shown that rats on a zinc deficient diet have significantly reduced levels of plasma vitamin A, RBP and hepatic RBP. The growth restriction that accompanies zinc deficiency due to reduced food intake also contributes to the decreased plasma vitamin A and RBP. However, zinc-deficient rats that are pair-fed to zinc-sufficient rats, have an even lower plasma vitamin A and RBP levels. Furthermore the decreased plasma RBP levels were more pronounced than that of total protein concentrations in zinc-deficient rats, suggesting a specific effect on RBP. The reduced hepatic RBP is thought to be due to depressed synthesis of RBP in zinc deficient rats probably because of a lower rate of nucleic acid and protein synthesis (Olson, 1991). It has also been recently suggested that the elevation of hepatic vitamin A in zinc deficiency may be, in part, secondary to a change in the activity of enzymes that regulate retinol degradation (Boron et al. 1988). Thus, activity of hepatic alcohol dehydrogenase, a zinc-dependent enzyme invoived in the conversion of retinol to retinaldehyde, has been found to be significantly reduced in zinc deficiency.

Stress

Stress of physical, psychological or pathological origin may influence levels of vitamin A status. Stresses such as in burn injury, surgical operations, fevers and infections have all been associated with low plasma levels of retinol and RBP. In thermal burn patients with burn surface area of up to 30%, serum vitamin A and RBP have been reported to be lower than normal; these changes appear to correlate closely with the severity of the trauma (Cynober et al., 1985). The decrease in plasma RBP levels is attributed either to visceral protein breakdown or to a redirection of the synthesis of individual proteins. In a study involving rats, prolonged immobilization-induced stress which simulated weightlessness, depressed the plasma retinol and RBP levels but the hepatic retinyl palmitate levels were higher than in pair-fed controls (Takase et al., 1992). The decrease in plasma retinol in this study is thought to be, at least in part, due to a decrease in mobilization of vitamin A from the liver. Stress situations are known to trigger secretion of hormones including glucagon, glucocorticoids and catecholamines. Administration of cortisone to normal rats markedly lowers the weights of the thymus and adrenals and reduces the concentrations of vitamin A in the plasma, liver, thymus and adrenals (Atukorala et al., 1981), suggesting that this hormone favors elimination of vitamin A from tissues. However, other studies show that administration of glucocorticoids enhances a release of vitamin A from hepatic stores with elevation of plasma vitamin A in rats (McGillvray, 1961). During in vitro studies with rat hepatoma cells cultured in serum-free medium, corticosterone and cortisol induced a 2-to 3-fold increase in accumulation of RBP (Borek et al., 1981). This suggests that these hormones may accelerate mobilization of vitamin A from the liver as observed in the in vivo study (McGillvray, 1961), possibly through an increase in RBP synthesis.

Disease

Studies examining plasma vitamin A, RBP amd TTR levels in patients with various disease conditions are abundant. These include: acute and chronic diseases of the liver (Smith and Goodman, 1971; McClain et al., 1979), kidney diseases (Smith and Goodman, 1971), measles (Sommer et al., 1986) and diabetes (Basu et al., 1989).

In a study by Smith and Goodman (1971), 63 patients with liver disease had plasma retinol, RBP and TTR levels which were all lower than normal. These low levels presumably reflect a reduced rate of hepatic production of the proteins (Goodman, 1984). A follow-up examination of the patients with hepatitis revealed that with progressive improvement of the disease, the plasma levels of vitamin A, RBP and TTR also improved.

Renal disorders are often accompanied by impaired vitamin A status. The kidneys play an important role in the catabolism of proteins. Once RBP has given up retinol in the target tissues, the resulting apoRBP has a reduced affinity for TTR. Due to its low molecular weight, the apoRBP rapidly undergoes glomerular filtration in the kidney (Peterson et al., 1974). In chronic renal disease of varying etiologies, Smith and Goodman (1971) found that plasma RBP and vitamin A levels were markedly elevated, while TTR levels remained normal. The plasma RBP levels in many of these patients were present in molar excess of TTR, suggesting impaired renal clearence of RBP. However, patients with tubular proteinuria have increased urinary RBP, showing that tubular reabsorption of RBP is impaired. In Japanese patients with chronic cadmium poisoning, large amounts of RBP were excreted in the urine. However, plasma concentrations of RBP of these patients were normal probably because the tubular reabsorption does not influence the glomerular filtration rate (Vahlquist et al., 1973).

Retinitis pigmentosa is an hereditary disease of the eye where the patient suffers night blindness, progressive visual field loss and eventually loss of the central vision (Massof and Finkelstein, 1993). Because of the similarity between the histopathology of the retina in retinitis pigmentosa (Bridges, 1984) and in vitamin A deficiency (Dowling and Wald, 1960), the link between the disease and vitamin A metabolism has been sought. However, controversy exists as to whether vitamin A supplementation slows the progression of the disease (Berson et al., 1993; Norton, 1993; Massof and Finkelstein, 1993), since patients have normal serum retinol and RBP levels (Bridges, 1984; Berson et al., 1993).

CONCLUSION AND OBJECTIVES OF THE PRESENT STUDY

Diabetes mellitus is known to affect nutritional status. Research on the relationship between diabetes and macronutrients particularly on carbohydrate and lipid metabolism is extensive.

However, very little work has been reported on the effect of diabetes on micronutrient metabolism. Reports discussed above suggest that vitamin A status in diabetes is of concern. The causes of the poor vitamin A status are not known but appear to be secondary to dietary intake. Possible factors may include an alteration in its absorption, mobilization from hepatic stores, availability of its carrier proteins, zinc metabolism and insulin availability. The biochemical evidence points to the impaired mobilization of vitamin A from its hepatic store into circulation. Thus it was hypothesized that diabetes is associated with depressed vitamin A status due to reduced availability of its carrier proteins. Using streptozotocin(STZ)-induced diabetic rats, this hypothesis was tested:

1. To identify a rat strain sensitive to STZ in terms of vitamin A status.
2. To determine the responses to vitamin A supplementation and pair-feeding in plasma, liver and retina.
3. To examine in vitro intestinal absorption of [^{3}H]retinol.
4. To determine the distribution of retinol carrier proteins in the plasma, liver and kidney.
5. To examine the responses of insulin administration or zinc supplementation on circulatory and hepatic vitamin A concentrations.

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2. STRAIN VARIATION IN VITAMIN A (RETINOL) STATUS OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

INTRODUCTION

Abnormal metabolism of vitamin A, as indicated by its decreased circulatory levels along with its carrier protein, retinol-binding protein (RBP), has been reported in patients with diabetes mellitus (Basu et al., 1989; Krempf et al., 1991; Martinoli et al., 1993). This is an important observation since chronic vitamin A deficiency (Oomen, 1974) and diabetes (Palmberg, 1977) share a common clinical consequence - i.e., blindness. Although the underlying mechanism for this clinical consequence is different in vitamin A deficiency and in diabetes, it is possible that retinopathy, precipitated by diabetes, can be further aggravated in the presence of a vitamin A deficiency. It is therefore important that the metabolism of vitamin A is further studied in experimental animals in order to obtain a better understanding of the relationship between diabetes and vitamin A metabolism.

In humans and rats, vitamin A is stored primarily in the liver as retinyl esters. The delivery to peripheral tissues requires hydrolysis of retinyl esters to retinol which is subsequently transported by RBP to the circulation; retinol thus becomes the major transport form of vitamin A with very little of its ester form in the circulation. However, there seem to be rat strain differences in vitamin A metabolism in response to certain treatments such as chronic alcohol consumption (Seifert et al., 1991). The present study was undertaken to examine vitamin A status in streptozotocin-induced diabetic rats of different strains. The objective being to identify a suitable rat strain which is similar to humans in terms of the metabolism of vitamin A in response to diabetes. This is particularly important because the metabolism of vitamin A appears to be different from one species or strain to another (Wilson et al., 1987; Seifert et al., 1991).

METHODS AND MATERIALS

Animals and diets

Male Wistar Furth (WF/NHsd), Sprague Dawley (Crl: CD®(SD)BR) and Wistar (Crl: (WI)BR) rats weighing 220-300g were obtained from Harlan Sprague Dawley Inc., Indiana, U.S.A and Charles River, Montreal, Canada. They were housed in stainless steel metabolic cages in a well-ventilated room maintained at 21^0 C and were on a 12-hour light-dark cycle. Diabetes was induced by a single intravenous injection of streptozotocin (STZ) (55 mg/Kg) (Upjohn, Kalamazoo, Michigan) dissolved in acetate buffer (pH 4.5). Control rats were injected with acetate buffer alone. Following injection of streptozotocin, animals displaying plasma glucose of greater than 18 mmol/L were considered diabetic. Plasma and urinary glucose concentrations were measured by glucose oxidase method using the Beckman Glucose Analyzer (Kadish et al., 1968). Semi-synthetic diet (Table 2-1) and water were supplied ad libitum to all the animals throughout a 4-week study period. Experimental protocols were reviewed and approved by the Animal Policy and Welfare Committee, University of Alberta.

A record of food intake, body weight, plasma glucose, urinary loss and urinary glucose was kept throughout the study. Animals were killed at the end of 4 weeks using carbon dioxide. Blood was collected in heparinized tubes; separated plasma was protected from light and stored at -20^0 C pending analysis. Livers were removed, cleaned and frozen immediately in liquid nitrogen.

Vitamin A determination

Plasma (Nierenberg and Lester, 1985) and liver (Frolik and Olson, 1984) vitamin A were assayed by high-performance liquid chromatography (HPLC) using retinyl acetate (Sigma Chemical Co., St. Louis, MO) as the internal standard. For extraction of retinol from plasma, 200 µL plasma samples were pipetted into microcentrifuge tubes. The internal standard, 200 ng/mL of retinyl acetate dissolved in acetonitrile was then added and vortexed for 15 seconds. A total of 250 µL of butanol:ethyl acetate (1:1) was added and then vortexed again for 60 seconds. Finally 150 µL of an aqueous solution of dipotassium monohydrogen phosphate (1.2 g/ml) was added, the solution vortexed for a further 30 seconds and then centrifuged at 9000 rpm for 1 minute, to separate the phases. The organic upper layer was injected into the HPLC. For each sample, duplicate aliquotes were extracted. All procedures were performed in dim light and solutions containing vitamin A were protected from light.

TABLE 2-1. Composition of the semi-synthetic diet[*]

Ingredient	(g/Kg)
Casein (vitamin free)	200
Corn starch	600
Non-nutritive bulk	50
Corn oil	100
AIN Vitamin-mix (with choline)[†]	12.5
AIN Mineral mixture[‡]	35
DL-Methionine	2.5

[*] Ingredients are from ICN Biomedicals Cleveland, Ohio.

[†] Retinyl acetate in the basal diet, 3.6 RE(12 IU)/g diet; vitamin A supplement, 36 RE(120 IU)/g diet. Vitamin mixture, g/Kg mixture: vitamin A acetate (500,000 IU/g), 1.98; vitamin D_2 (850,000 IU/g), 0.138; vitamin E acetate (500 IU/g), 11.0; ascorbic acid, 49.5; Inositol 5.5; choline bitatrate 222.7; menadione, 2.475; p-aminobenzoic acid, 5.5; niacin, 4.675; riboflavin, 1.1; pyridoxine HCl, 1.1; thyamine HCl, 1.1; D-calcium pantothenate, 3.3; biotin, 0.022; folic acid, 0.099; vitamin B-12, 0.00149.

[‡] Mineral mixture, g/Kg mixture: calcium phosphate diabasic, 500; sodium chloride, 74; potassium citrate monohydrate, 220; potassium sulfate, 52; magnesium oxide, 24; manganous carbonate (43-48% Mn), 3.5; ferric citrate (16-17% Fe), 6; zinc carbonate (70% ZnO), 1.6; cupric carbonate (53-55% Cu), 0.3; potassium iodate, 0.01; sodium selenite 0.01; chromium potassium sulfate, 0.55.

Liver samples were homogenized in 9 volumes (wt/v) of water in a polytron homogenizer for 20 seconds. The homogenate was then saponified with equal an volume of 5% potassium hydroxide in methanol for 1 hour at 50^0 C. The saponified liver samples were extracted with hexane. Hexane was removed under vacuum in a Savant spin evaporator. Residues were dissolved in acetonitrile and then injected into the HPLC.

The HPLC equipment used consisted of a Waters 600E Multidelivery system pump (Waters Associates, Milford, MA); a Waters 486 tunable ultraviolet/visible absorbance detector; and a Waters computer recorder equipped with a Millenium software integrator. A reverse phase, C18 column (Whatman partisil SODS-3) was used with an isocratic solvent system, methanol: water (95:5) at a flow-rate of 1.5 ml/minute. All solvents used were HPLC grade and the mobile phase was filtered and de-gassed before each run. Detection was by UV absorption at 325 nm, and quantification was performed by comparison of ratio of peak areas produced by the injection of known amounts of retinol and an amount of internal standard equal to that in the samples.

Statistical analysis

Data were tabulated to give means and standard error of the mean using SAS computer program (SAS Institute, Cary, NC). Data for the three strains of rats were compared by using two way analysis of variance (ANOVA) (Steel and Torrie, 1980). When significant differences were detected by ANOVA, the appropriate comparisons were made by Scheffe's test. To determine significant differences between group means, Student's t-test was used. The level of significance was set at $p < 0.05$.

RESULTS

All three rat strains exhibited the characteristic signs of experimentally induced diabetes such as elevated plasma glucose, increased urinary glucose, urinary volume and water intake, within 48 hours of administration of a single intravenous dose of streptozotocin. The differences in mean plasma and urine concentrations of glucose between STZ-treated rats and their corresponding controls at the end of 4 weeks, are shown in Table 2-2.

TABLE 2-2. Effect of diabetes on the characteristics of rats of different strains†

Strain	Food intake (mg/g Bwt)	Body wt change (g)	Liver weights (g)	Plasma glucose (mmol/L)	Urinary glucose (g/24 hours)
Wistar Furth					
Control	45 ± 0.31	53 ± 2.2	7.0 ± 0.17	6.0 ± 0.24	0.008 ± 0.001
Diabetic	101 ± 3.6*	-76 ± 6.9*	7.1 ± 0.39	25.0 ± 0.8*	10.0 ± 1.4*
Sprague Dawley					
Control	54 ± 2.2	95 ± 9.3	11.1 ± 0.27	6.7 ± 0.2	0.019 ± 0.007
Diabetic	122 ± 2.8*	-20 ± 12.3*	11.3 ± 0.51	19.3 ± 3.1*	16 ± 2.7*
Wistar					
Control	54 ± 0.67	117 ± 6.3	13.3 ± 0.49	6.4 ± 0.4	0.047 ± 0.02
Diabetic	115 ± 3.7*	-22 ± 2.1*	11.3 ± 0.33	19.8 ± 2.4*	12.5 ± 1.31*

† Results are expressed as mean ± SEM of six rats.

*Control verses diabetic differ significantly at $p<0.001$

All the STZ-induced animals weighed less than the controls even though their daily food intake was higher than that of the controls. Plasma vitamin A concentrations were significantly lower in diabetic Sprague Dawley ($p < 0.02$) and Wistar ($p < 0.0001$) rats, when compared to their corresponding non-diabetic controls (Table 2-3). Plasma retinol levels in diabetic Wistar Furth rats, however, were unaffected.

TABLE 2-3. Effect of STZ-induced diabetes on the plasma retinol concentrations in rats of different strains †

	Retinol, μmol/L		
Strain	Control	Diabetic	p-value *
Wistar Furth	0.84 ± 0.03^{a}	0.93 ± 0.11^{c}	0.07
Sprague Dawley	1.09 ± 0.20^{ab}	0.67 ± 0.30^{c}	0.02
Wistar	1.33 ± 0.20^{b}	0.77 ± 0.18^{c}	0.0001

† Results are expressed as mean ± SEM of six rats.

Statistical comparisons among the strains are shown using superscripts (a, b, c). Within a column, values not sharing a common superscript letter differ significantly ($p < 0.05$) as determined by Scheffe's test.

*Comparison between control and diabetic animals.

Unlike the plasma levels, hepatic concentrations of vitamin A were markedly increased in the presence of diabetes in all three strains of rats as shown in Table 2-4.

TABLE 2-4. Effect of STZ-induced diabetes on the hepatic vitamin A concentrations in rats of different strains†

	Total vitamin A, µmol/g		
Strain	Control	Diabetic	p-value *
Wistar Furth	0.57 ± 0.04[a]	0.85 ± 0.08[b]	0.01
Sprague Dawley	0.46 ± 0.06[a]	0.74 ± 0.04[b]	0.004
Wistar	0.57 ± 0.03[a]	0.77 ± 0.04[b]	0.001

† Results are expressed as mean ± SEM of six rats.

Statistical comparisons among the strains are shown using superscripts (a, b). Within a column, values not sharing a common superscript letter differ significantly ($p<0.05$) as determined by Scheffe's test.

*Comparison between control and diabetic rats.

DISCUSSION

All three strains of rats including Wistar Furth, Sprague Dawley and Wistar became hyperglycemic after STZ administration. The plasma levels of vitamin A however, were only affected in the Sprague Dawley and Wistar but not in Wistar Furth rats. The plasma retinol concentrations were significantly lower in the Sprague Dawley ($p < 0.02$) and Wistar ($p < 0.0001$) diabetic as compared to the control animals. This finding is in agreement with earlier human studies in which the plasma retinol levels were found to be significantly reduced in the IDDM patients than in non-diabetic controls (Basu et al., 1989; Krempf et al., 1991; Martinoli et al., 1993). However, diabetic Wistar Furth rats responded differently from Wistar or Sprague Dawley rats in that their plasma retinol levels remained at control values. The reason for this difference is not clearly understood, however, it is known that Wistar Furth rats are inbred while Sprague Dawley and Wistar are of outbred strains. The fact that there was no difference in circulatory vitamin A between control and diabetic Wistar Furth suggests that vitamin A metabolism may be strain-dependent. This has been observed in another study involving chronic alcohol consumption. The difference was in the liver vitamin A concentrations which were significantly reduced in one strain, WAG/Rij but were not affected in another, BN/BiRij as compared to their corresponding control rats (Seifert et al., 1991).

In contrast to plasma retinol, hepatic vitamin A was higher in the diabetic than in the control animals regardless of their strain. This may be explained in part, by the increased food consumption (>50%) by the diabetic animals. It has been reported that the hepatic storage of vitamin A is proportional to its intake (Hicks et al., 1984). It seems therefore, possible that the increased liver vitamin A may be due to an increased consumption of the vitamin. Plasma RBP levels have been shown to be decreased in type I diabetic patients (Basu et al., 1989; Krempf et al., 1991; Martinoli et al., 1993). This may suggest that the STZ-induced diabetes-associated increase in hepatic vitamin A may be caused by its decreased mobilization from its storage to circulation. However, intestinal absorption of lipids has been reported to be enhanced in diabetic animals (Thomson, 1983). Since vitamin A is lipid-soluble, its absorption may be increased in the presence of diabetes, resulting in an increased hepatic storage. Thus, more studies are required to further elucidate the effect of diabetes on the metabolism of vitamin A. For these studies, Wistar will be the choice of rat strain, because rats of this strain with diabetes are most sensitive in terms of their circulatory retinol status, and this response is parallel to the observations made in diabetic patients (Basu et al., 1989)

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3. EFFECTS OF VITAMIN A SUPPLEMENTATION AND PAIR-FEEDING ON VITAMIN A STATUS IN STREPTOZOTOCIN-INDUCED DIABETES IN RATS

INTRODUCTION

Diabetes mellitus is a chronic disease characterized by abnormality in the metabolism of carbohydrate, lipid, and protein. Vitamin A metabolism is also known to be altered as indicated by decreased concentrations plasma retinol and its carrier protein, retinol-binding protein in insulin dependent-diabetic patients (Basu et al., 1989; Krempf et al., 1991; Martinoli et al., 1993). Further, Wako et al., (1986) showed that reduced plasma levels of retinol are accompanied by increased concentrations of plasma retinyl esters in non-insulin dependent diabetic patients, suggesting increased hepatic storage of vitamin A. Despite the decreased plasma levels of retinol, liver concentrations of vitamin A have been found to be increased in streptozotocin-induced diabetic rats (Basu et al., 1990). The reason for the subnormal circulatory levels of vitamin A despite its increased hepatic storage in diabetes is not fully understood.

Liver is the main storage site of vitamin A as retinyl esters and it plays a dynamic role in vitamin A metabolism. It generates retinol from the stored retinyl esters as well as in the synthesis of retinol binding protein which is essential for the transport of retinol to extrahepatic tissues. Under normal conditions, hepatic storage of vitamin A is a reflection of its intake (Hicks et al., 1984), but its plasma levels are regulated such that it is constant, independent of its dietary intake. However, in vitamin A deficiency, hepatic storage is depleted so that plasma levels fall and supplementation becomes necessary.

Using streptozotocin(STZ)-induced male Wistar (Crl: (WI)BR) diabetic rats, the present study was undertaken to examine the effect of vitamin A supplementation and pair-feeding on the availability of vitamin A in the plasma, liver and retina of the eye. The purpose was to examine any reversal in plasma depressed retinol levels; and if elevated hepatic vitamin A concentrations are due to an increased vitamin A intake.

MATERIALS AND METHODS

Animals and tissue collections

Male Wistar rats (Crl: (WI)BR), weighing 250-300 g were obtained from Charles River, Montreal, Canada. They were housed in stainless steel cages in a well-ventilated room maintained at 21^0 C and were on a 12-hour light-dark cycle. Diabetes was induced by intravenous injection of STZ as described in chapter 2. Following injection with STZ, animals displaying plasma glucose of greater than 18 mmol/L were considered diabetic. Plasma glucose concentrations were measured by glucose oxidase method using the Beckman Glucose Analyzer (Kadish et. al., 1968).

Three experimental groups of animals were studied for a period of 4 weeks. In experiments 1 and 2, control and diabetic animals were given free access to a semisynthetic diet (Table 2-1, chapter 2) either with or without vitamin A supplementation. In experiment 3, individual rats were pair-fed the semisynthetic diet so that each diabetic rat consumed a daily weight of food equal to that consumed by weight-matched control rat on the previous day, thus each pair of control and diabetic animals had received the same amount of vitamin A per day. Vitamin A supplemented groups were given free access to the semisynthetic diet but supplemented with 10 times more retinyl acetate (36 RE/g diet). All animals were allowed free access to water and a record of food intake and body weight was kept throughout the study. Animals were killed at the end of 4 weeks using carbon dioxide. Blood was collected in heparinized tubes; separated plasma was protected from light and stored at -20^0 C, pending analysis. Livers were removed, cleaned and frozen immediately in liquid nitrogen. Retina of the eye was isolated by the method of Uehara et al. (1989). The corneas were pierced and dissected with the tip of a razor blade. Using forceps, slight pressure was applied to the eye cup enabling the lens and vitreous body to be easily extracted. While the lens was extruded, the retina which is connected to the lens by the zonular fibers came out with lens. The retina was then detached from the lens and was washed two to three times with 2 to 3 drops of cold saline solution (4^0 C) and frozen in liquid nitrogen.

Vitamin A analysis

Plasma (Nierenberg and Lester, 1985) and liver (Frolik and Olson, 1984) vitamin A were assayed by HPLC using retinyl acetate as the internal standard as described in chapter 2. The saponified liver samples were extracted with hexane but free retinol in the liver was extracted without saponification. Retina was analysed for 11-cis retinal with a modification of the method outlined by Suzuki et al. (1988). Samples were homogenized then mixed with 37% formaldehyde, isopropanol and hexane. The hexane was then collected and evaporated to dryness with nitrogen and redissolved in HPLC mobile phase (3% dioxane in hexane), prior to analysis. Detection was by UV absorption at 360 nm, and quantification was performed using a standard curve determined with authentic 11-cis retinal.

Statistical Analysis

Data were tabulated to give means and standard error of the mean using SAS computer program (SAS Institute, Cary, NC). To determine differences between group means, the Student's t-test was used for the non-supplemented and the vitamin A supplemented groups; and the Paired-t test was used for the pair-fed group (Steel & Torrie, 1980). The upper level of significance was preset at $p < 0.05$.

RESULTS

The STZ-induced diabetic rats given free access to the semisynthetic diet with (Table 3-1) or without supplementation (Table 3-2) of vitamin A exhibited elevated plasma glucose concentrations with no difference between the two groups. This hyperglycemia was accompanied by glycosuria and this became manifested within 48 hours, and persisted throughout the 4 week followup period. Even though they consumed significantly more food, the diabetic animals initially lost weight and then gained weight slower than did the corresponding controls. The weekly mean body weight changes and mean daily food intakes of all the groups in the three experiments are shown together in Figures 3-1 and 3-2, respectively. Despite polyphagia that resulted in a 48% greater intake of vitamin A, plasma retinol concentrations were significantly lower in the diabetic animals than in the controls ($p < 0.003$) (Table 3-1). However, the hepatic concentrations of vitamin A were markedly greater in the diabetic animals ($p < 0.0001$).

TABLE 3-1. Vitamin A status of nonsupplemented control and diabetic rats*

	Control	Diabetic	p-value
Total body weight gain (g)	185 ± 13	24 ± 16	0.0001
Liver weights (g)	15.8 ± 0.57	14.3 ± 0.61	0.09
Plasma glucose (mmol/L)	6.00 ± 0.24	21.6 ± 1.91	0.0001
Food intake (g/day)	23.4 ± 0.94	34.0 ± 0.95	0.0001
Vitamin A intake (RE/day)	84.2 ± 3.40	122 ± 3.40	0.0001
Plasma retinol (μmol/L)	1.35 ± 0.10	0.91 ± 0.05	0.003
Hepatic vitamin A (μmol/g liver)†	0.50 ± 0.01	0.99 ± 0.05	0.0001

*Results are expressed as mean ± SEM (n = 6). Diets contained 3.6 RE retinyl acetate/g).

†Hepatic vitamin A includes free retinol + retinyl esters.

To try and correct for this decreased plasma retinol concentration, in experiment 2, the diabetic rats were fed a diet supplemented with vitamin A (36 RE/g) which was 10 times of what was present in the basal diet. Even after 4 weeks of this supplementation, the circulatory level of retinol in diabetic animals were lower ($p < 0.05$) while hepatic concentrations increased at this supplemental level as compared to the control animals ($p < 0.0001$) (Table 3-2).

TABLE 3-2. Effect of vitamin A supplementation on the plasma and hepatic concentrations of vitamin A in control and diabetic rats *

	Control	Diabetic	p-value
Total body weight gain (g)	185 ± 16	53 ± 13	0.0001
Liver weights (g)	16.7 ± 0.99	14.7 ± 0.66	0.12
Plasma glucose (mmol/L)	6.20 ± 0.14	22.7 ± 3.15	0.0004
Food intake (g/day)†	21.9 ± 0.73	34.7 ± 2.19	0.0002
Vitamin A intake (RE/day)	871 ± 29.7	1377 ± 86.1	0.0002
Plasma retinol (μmol/L)	1.18 ± 0.07	0.96 ± 0.08	0.05
Hepatic vit A (μmol/g liver)‡	2.64 ± 0.18	5.21± 0.42	0.0001

*Results are expressed as mean ± SEM (n = 6). †Diets were supplemented with vitamin A (36 RE retinyl acetate/g). ‡Hepatic vitamin A includes free retinol and retinyl esters.

In experiment 3, the pair-fed diabetic and control rats were fed isocalorically a semisynthetic diet (Table 2-1) with the same amount of vitamin A for 4 weeks. These pair-fed animals had similar hepatic concentrations of the vitamin and yet plasma concentrations remained significantly lower in the diabetic rats ($p < 0.01$) (Table 3-3). The free retinol concentration in the liver however was significantly higher in the diabetic than in the control animals ($p < 0.001$). In the retina, the content of 11-cis retinal, an important constituent of rhodopsin, was found to be significantly lower in the diabetic animals than in the controls ($p < 0.05$).

TABLE 3-3. Vitamin A status of pair-fed control and diabetic rats*

	Control	Diabetic	p-value
Body weight gain (g)	159 ± 12	25 ± 19	0.0001
Liver weights (g)	13.7 ± 0.66	12.0 ± 0.84	0.11
Food intake (g/day)†	21.0 ± 0.95	21.0 ± 0.85	0.88
Vitamin A intake (RE/day)	75.7 ± 2.91	75.6 ± 2.94	0.98
Plasma retinol (μmol/L)	1.16 ± 0.06	0.65 ± 0.08	0.01
Hepatic vitamin A (μmol/g liver)‡	0.48 ± 0.02	0.51 ± 0.03	0.11
Free retinol (μmol/g liver)	0.018 ± 0.002	0.067 ± 0.008	0.001
11-cis retinal (pmol)	52.8 ± 16.5	22.1 ± 3.3	0.05

*Results are expressed as mean ± SEM (n = 6). †Diets contained 3.6 RE retinyl acetate/g.

‡ Hepatic vitamin A includes free retinol + retinyl esters.

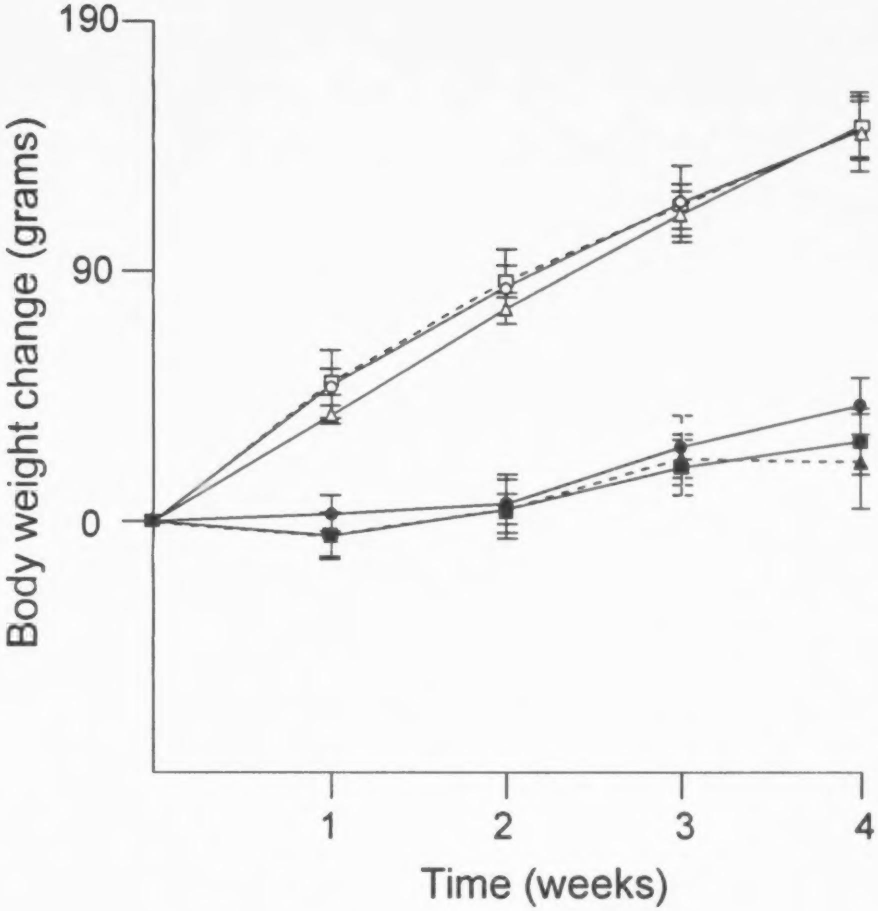

FIGURE 3-1. Mean weekly body weight change of ad libitum fed unsupplemented control,-□- ; and diabetic, -■-;vitamin A supplemented control, -○-; and diabetic, -●-;and pairfed control,-△-; and diabetic, -▲- rats. Each point on the graph represents mean±SEM of six animals.

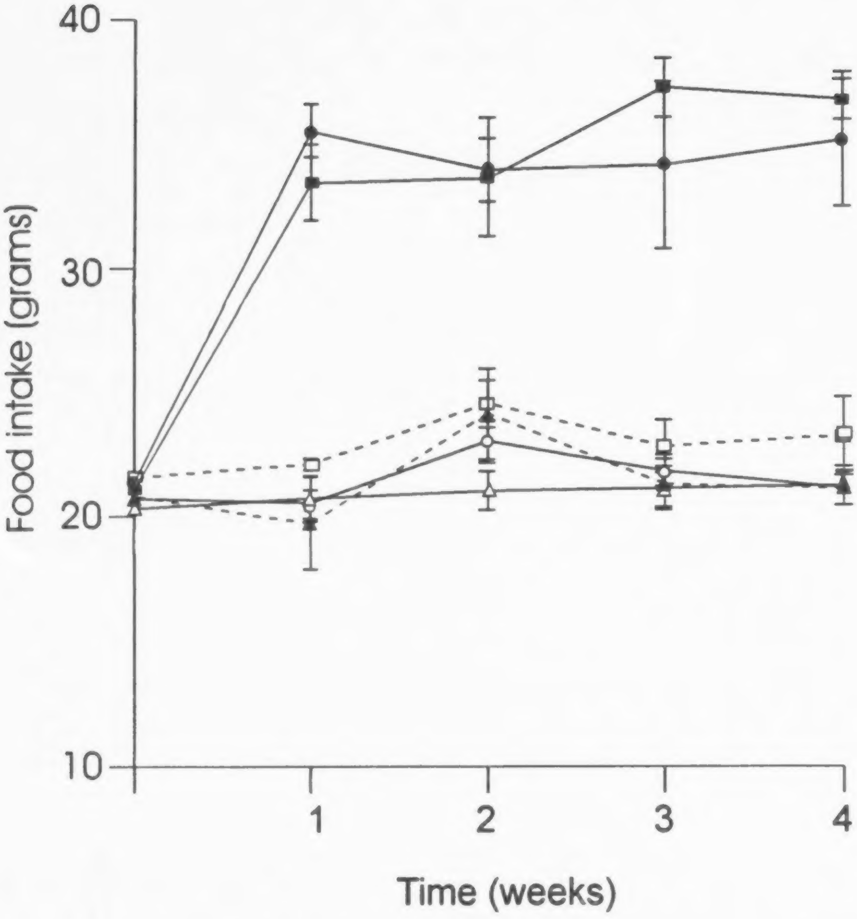

FIGURE 3-2. Mean daily food intake of ad libitum fed unsupplemented control,-□- ; and diabetic, —■— ;vitamin A supplemented control, —○—; and diabetic, —●—;and pairfed control,—△—; and diabetic, -▲- rats. Each point on the graph represents mean±SEM of six animals.

DISCUSSION

The results presented here clearly demonstrate that rats made diabetic with STZ have significantly reduced concentrations of plasma retinol. The results are in agreement with earlier human studies in which the plasma retinol concentrations were shown to be significantly lower in the diabetic patients than in the non-diabetic controls (Basu et al., 1989, Krempf et al., 1991; Martinoli et al., 1993). Despite the lower plasma retinol concentrations, liver concentrations of total vitamin A were significantly elevated in the diabetic rats than in the controls. These animals consumed 48% more food than the corresponding controls. Since the hepatic stores of vitamin A are thought to be proportional to daily intake (Hicks et al., 1984), it seems possible that the increased intake of vitamin A by the diabetic animals account, at least in part, for their elevated hepatic concentrations. However, the fact remains that the diabetes-associated reductions in plasma retinol levels were not due to the depletion of hepatic stores. In order to determine if decreased plasma retinol in diabetic animals could be increased to normal concentrations, diabetic animals were fed a diet which contained 10 times the basal diet of vitamin A (36 RE/g) for 4 weeks. At this augmented intake, the plasma concentrations of vitamin A were lower in the diabetic animals, while the hepatic levels increased as compared to the control group.

In the pair-feeding experiment, in which the control and diabetic rats were fed equal amounts of vitamin A, the plasma retinol remained low in the diabetic animals. However, unlike the diabetic animals given free access to food, the total hepatic concentration of vitamin A in the pair-fed diabetic rats was similar to that in the control animals. Since there was no difference in the hepatic concentration of total vitamin A between the control and diabetic pair-fed animals, it is plausible that absorption of vitamin A is not affected by diabetes. It may be argued that in the present study involving diabetic rats which were pair-fed to non-diabetic controls, that decreased plasma did not result in an increased hepatic concentrations of the vitamin. Vitamin A is predominantly stored in the liver and its amount present in the circulation accounts for less than 1% of the total body pool size of the vitamin (Ostrowski, et al., 1989). The diabetes associated reductions in plasma retinol levels may not be enough to have an appreciable effect on the hepatic size of the vitamin, especially over a period of 4 weeks.

The results from this study suggest that there may be an impairment in the availability of vitamin A from hepatic store in the presence of diabetes. Vitamin A is stored in the liver as retinyl ester (Blomhoff et al., 1991). It is hydrolysed by retinyl ester hydrolase, and carried as free retinol to the circulation and subsequently to the target tissues by RBP (Goodman, 1974). It is important to note that although the pair-fed diabetic and control animals showed no difference in their hepatic concentrations of vitamin A (i.e., free retinol + retinyl ester), the diabetic animals exhibited an increased level of free retinol. It is, therefore, unlikely that the availability of vitamin A in diabetes is affected because of a decreased hepatic hydrolysis of the retinyl esters. An increase in hepatic free retinol levels accompanied by depressed circulatory retinol levels in the diabetic animals suggest that there may be an impaired mobilization of the vitamin from its hepatic stores into the circulation possibly due to inadequate synthesis of RBP in the liver. Studies in human diabetic subjects indeed show that there is paralleled decrease in plasma RBP with a decrease in plasma retinol (Basu et al., 1989; Krempf et al., 1991; Martinoli et al., 1993).

Lowered levels of plasma vitamin A have been linked with deficiency of zinc which is required for the synthesis of RBP (Smith, 1982). Hyperzincuria is a common feature of diabetes (Lau and Faila, 1984; Kinlaw et al., 1983), which may lead to zinc deficiency. It has been shown that in situations where abnormal dark adaptation is accompanied by poor zinc status such as in alcoholic cirrhosis, vitamin A supplementation alone does not improve the poor dark adaptation (Morrison et al., 1978), however normalization is accomplished when vitamin A was supplemented with zinc. It has further been shown that STZ-induced diabetes is associated with depressed protein synthesis in the liver (McNurlan and Garlick, 1981). It is therefore, possible that synthesis of RBP may be affected by secondary abnormalities in the metabolism of protein and zinc in the diabetic state.

Vitamin A is important in the visual cycle where 11-cis retinal is utilized by the photoreceptors (Bok, 1990). This involves a series of events that begin with photobleaching of rhodopsin to form opsin and all-trans-retinal, the production of various retinol derivatives, the regeneration of 11-cis retinal and ultimately, the regeneration of the photopigment itself. The present study also revealed that the retina of the eye of the diabetic rats is associated with reduced contents of 11-cis retinal when compared with those of the non-diabetic controls. This depressed retinal status may be a consequence of the decreased metabolic availability of vitamin A as indicated by its elevated hepatic and depressed circulatory levels.

The conclusion from the present study is that STZ-induced diabetes is associated with depressed vitamin A status as shown by the decreased levels of plasma retinol and 11-cis retinal in the retina. The mechanism for this abnormality is not yet fully understood. Future work needs to look at the effect of diabetes on the absorption and transport of the vitamin. It is important to note that the decreased levels of plasma retinol were not improved by vitamin A supplementation. The depressed plasma retinol levels and subsequently its unavailability to the retina may further aggravate the complication found in the retina that is often associated with the diabetic state. Since vitamin A supplementation resulted in a further increase of the vitamin in the liver it may also lead to hepatoxicity. The effects observed in this study were seen only after 4 weeks of diabetes. However, in the clinical situation, diabetes is a chronic condition, hence the results of the study are of importance.

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4. INTESTINAL ABSORPTION OF VITAMIN A IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

INTRODUCTION

Diabetes mellitus has been reported to be associated with biochemical evidence of vitamin A deficiency, as indicated by its decreased plasma levels along with its carrier, retinol-binding protein (RBP) (Basu et al., 1989; Basu et al., 1990; Krempf et al., 1991; Martinoli et al., 1993). Using streptozotocin (STZ)-induced diabetic rats, it has been shown that despite their markedly increased daily food consumption, the plasma retinol levels remain low while hepatic concentrations elevated, when compared with those of the non-diabetic control animals (Basu et al., 1990). The low circulatory retinol in diabetic animals appears to remain unchanged even when they are fed a diet supplemented with 10 times the basal level of vitamin A (Chapter 3); the liver concentrations of the vitamin are, however, proportionally increased in these animals. A decrease in 11-cis retinal (an important component of the visual pigment, rhodopsin) content has also been noted in the retina of the diabetic rats (Chapter 3); this may be a consequence of metabolic unavailability of retinol.

The availability of vitamin A appears to be of concern in diabetes, but the underlying cause is far from being understood. Diabetic rats store more vitamin A in the liver while their circulatory levels are lower than the control animals (Chapter 3). The increased liver levels of vitamin A may be attributed, at least in part, to an increased food intake by the diabetic animals. However, an enhanced absorption of the vitamin cannot be ruled out. Intestinal uptake of lipids is known to be enhanced in the presence of diabetes (Caspary, 1973; Thomson, 1980; 1983a; 1983b). Vitamin A is lipid soluble but its absorption in diabetic animals has not been studied. Vitamin A is present in the diet as retinyl esters or β-carotene. Dietary retinyl esters are hydrolysed to retinol (Fernandez and Borgstrom, 1990) within the intestinal lumen and retinol is then absorbed by passive, carrier-mediated process into the enterocyte (Hollander and Muralidhara, 1977; Said et al., 1988). On entry into the enterocyte, retinol is then reesterified and incorporated into chylomicrons which facilitate transport into the liver for storage (Fernandez and Borgstrom, 1990). The delivery of the vitamin to peripheral tissues requires the hydrolysis of the retinyl esters to retinol which is then transported by RBP into circulation where it binds with transthyretin (TTR) (Goodman, 1974).

The present study was undertaken to examine the in vitro uptake of retinol into the jejunum and ileum of diabetic rats.

MATERIALS AND METHODS

Animals and diets

Male Wistar rats, weighing 250-300 g were obtained from Charles River, Montreal, Canada. They were housed in stainless steel cages in a well-ventilated room maintained at 21^0 C and were on a 12-hour light-dark cycle. Diabetes was induced by single intravenous injection of streptozotocin (STZ) as described in chapter 2. Following injection with STZ, animals displaying plasma glucose of greater than 18 mmol/L were used in the study. Following induction of diabetes, diabetic and control animals were fed ad libitum a semisynthetic diet (Table 2-1, Chapter 2). All animals were allowed free access to water and a record of food intake and body weight was kept throughout 4 weeks of study.

Probe and Marker compounds

[^{14}C]polyethylene glycol was used as supplied by the manufacturer (New England Nuclear, Boston, MA) to measure the adherant intestinal mucosal fluid volume. Unlabeled and [^{3}H]retinol were obtained from Sigma Chemical Co. (St. Louis, MO) and from New England Nuclear, respectively). All-trans retinol was solubilized in 10 mM taurodeoxylic acid in Kreb's solution.

Tissue preparation

Control and diabetic animals were sacrificed by the injection of sodium thiopentol. A 15 cm length of proximal and distal intestine was rapidly removed and rinsed gently with 50 ml of cold saline, as described elsewhere (Thomson, 1980). The intestine was opened along its mesentric border and the mucosal surface was carefully washed with cold saline to remove visible mucus and debris. Pieces of intestine were cut from segments and the tissue was mounted as flat sheets in the transport chambers. The chambers and mounted tissue discs were placed in preincubation beakers containing a micellar solution prepared in oxygenated Krebs-Ringer phosphate buffer (20 mM NaH_2PO_4, 125 mM NaCl, 4.93 mM KCl, 1.23 mM $MgSO_4$, 0.85 mM $CaCl_2$, and 10 mM glucose, pH 6.5) at 37^0 C for 15 minutes to allow the tissue to equilibrate at this temperature. The transport chambers were then transferred to other incubation beakers containing [^{3}H]retinol. Preincubation and incubations were mixed at identical stirring rates with circular magnetic bars with the stirring rates precisely adjusted by means of a strobe light. Stirring rates were reported in revolutions per minute and a rate of 600 rpm was selected to achieve a low effect resistance of the intestinal unstirred water layer.

Determination of uptake rates

After preincubation, the chambers with the tissues were transferred to other beakers containing [^{14}C]polyethylene glycol and [^{3}H]retinol in oxygenated Krebs-Ringer phosphate (pH 6.5 at 37° C). Following incubation of the tissue discs in the labeled solutions for 6 minutes, each experiment was terminated by removing the chamber and quickly rinsing the tissue in cold saline for approximately 5 seconds. Exposed mucosal tissue was then cut out of the chamber with a circular steel punch, placed on glass slides, and dried overnight in an oven at 55° C. The dry weight of tissue was determined, the tissue sample was saponified with 0.75N NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes.

In samples of intestine not used for uptake studies, the mucosa was scraped, and the percentage of the wall of the intestine comprised of mucosa was determined. The weight of the mucosa in the samples used to measure uptake was determined by multiplying the dry weight of the mucosa, times the percentage of the intestinal wall comprised of mucosa. The rate of uptake of retinol was expressed as the nanomole taken up into the mucosa per minute per 100 mg dry weight of tissue (nmol/100 mg mucosa/min). All data were expressed as means ± standard error of the mean. Significant differences between control and diabetic group were analyzed using the Student's t-test and regression analysis.

RESULTS

Animal Characteristics

The STZ-induced diabetic rats exhibited the characteristic signs of diabetes such as elevated blood glucose, increased urinary excretion and water intake. Despite their increased food intake, these animals had a decreased body weight gain, compared to the non-diabetic controls (Table 4-1). The liver weights relative to body weights as well as the total weights of the jejunum and the ileum were also significantly ($p < 0.05$) higher in the diabetic than in the control animals, while no difference in the percentage of the intestinal wall comprised of mucosa was observed between the two groups (Table 4-1).

TABLE 4-1. Effect of diabetes on characteristics of animals†

Characteristic	Control	Diabetic
Food consumption gm/day	23.4 ± 0.45	42.3 ± 1.02***
Weight change (gm)	151 ± 4.36	73.0 ± 6.77***
Liver weights % body weight	4.63 ± 0.09	5.08 ± 0.16*
Jejunum Total weight (mg/cm)	14.0 ± 0.47	18.4 ± 0.73*
% comprised of mucosa	53.7 ± 3.97	50.3 ± 3.44
Ileum Total weight (mg/cm)	13.1 ± 0.92	18.7 ± 1.28*
% comprised of mucosa	50.4 ± 3.57	49.4 ± 3.72

† Results are expressed as mean ± SEM of six rats. * $p < 0.05$, *** $p < 0.0001$, diabetic verses control.

Retinol uptake

The uptake of retinol into the jejunum and the ileum as a function of time was determined at a concentration of 24 μM. The initial rate of jejunal and ileal uptake of retinol was linear up to 7 minutes, and thereafter became curvilinear (Figure 4-1). No difference in the rate of intestinal uptake of retinol was observed between the diabetic versus the control animals. The concentration of retinol was varied from 1 to 24 μM, and uptake was determined for 6 minutes. A linear relationship was noted between the concentration of retinol and uptake into both the jejunum and ileum

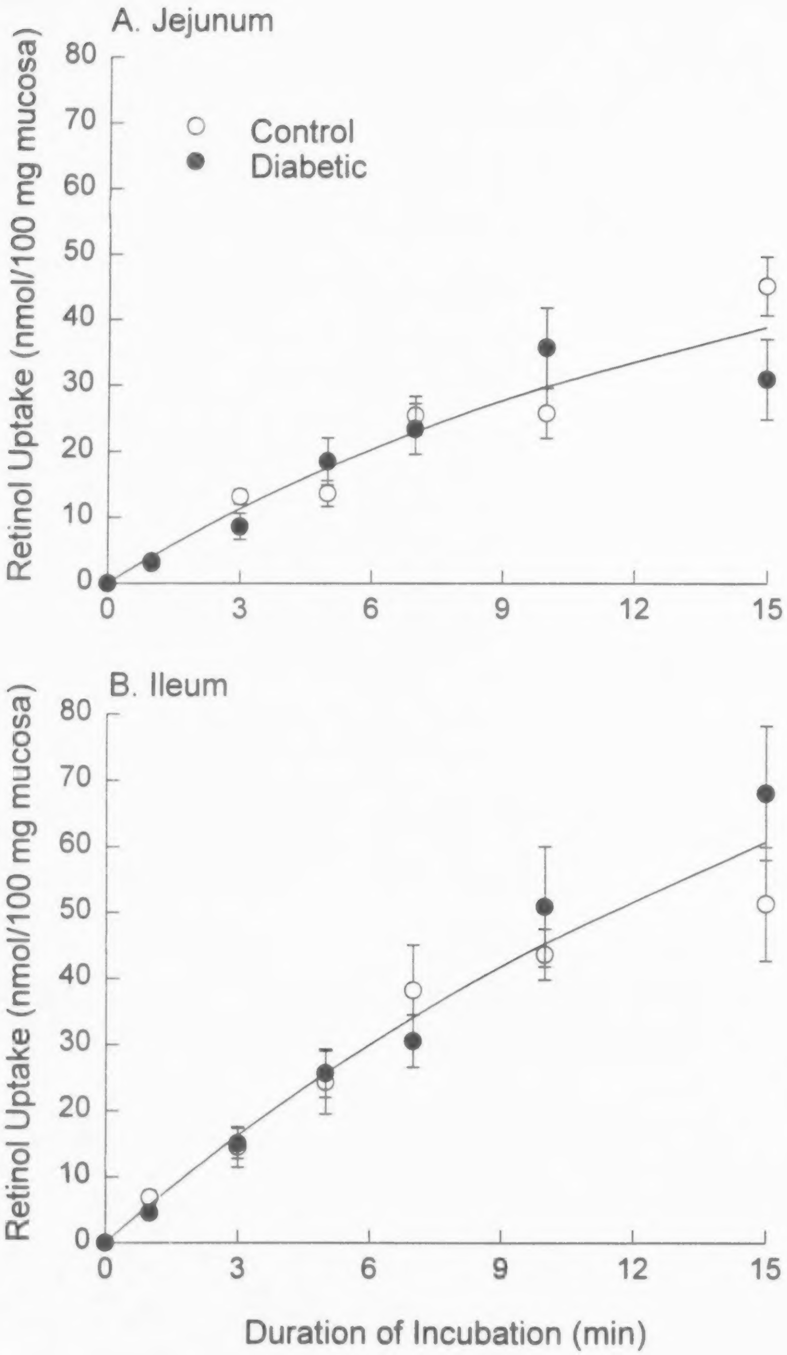

Figure 4-1: Effect of diabetes on the uptake of retinol into the jejunum and ileum at different incubation times. Each point represents the mean ± SEM of the values of six rats.

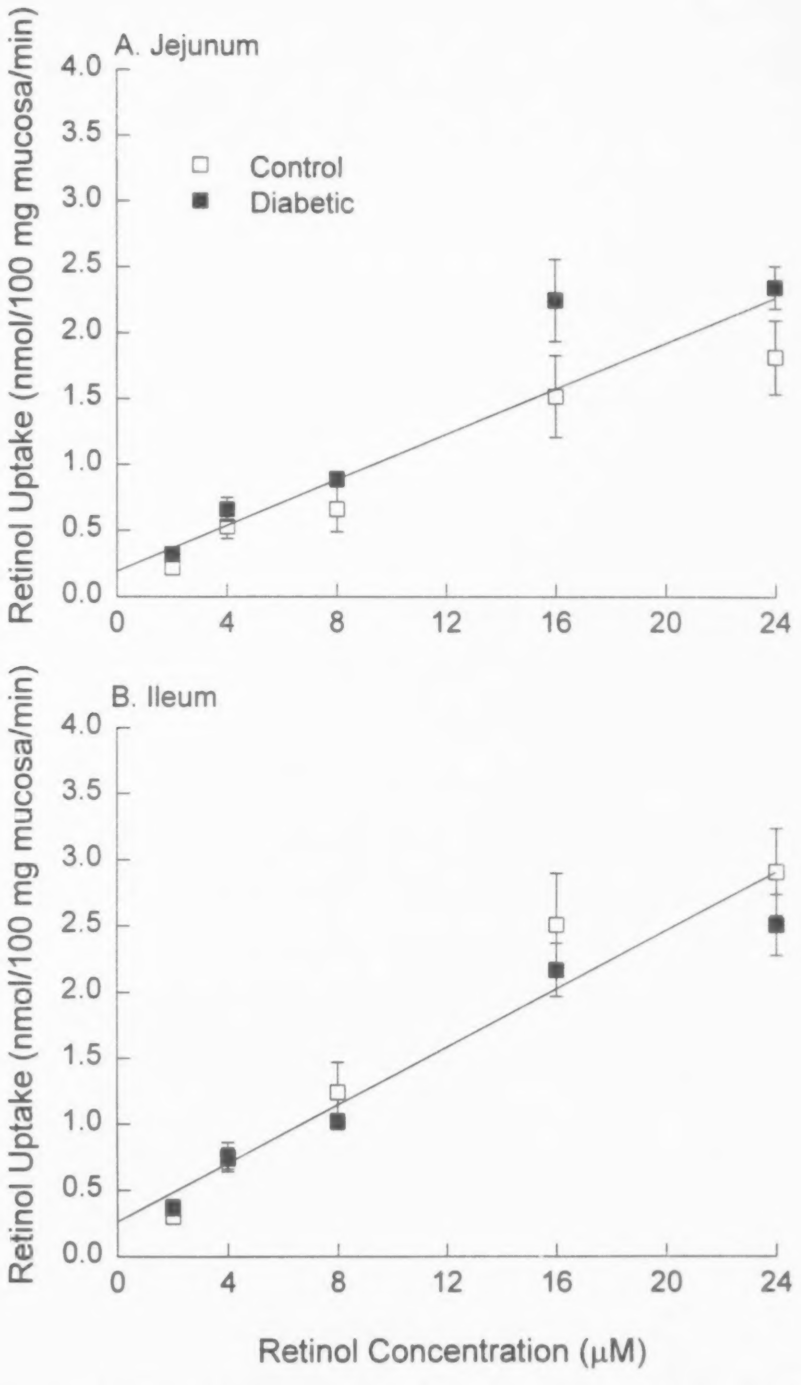

Figure 4-2: Effect of diabetes on the uptake of retinol into the jejunum and ileum at different concentrations. Each point represents the mean ± SEM of the values of six rats.

(Figure 4-2), again there was no appreciable difference in the uptake of retinol between the diabetic and control animals.

DISCUSSION

It has been shown that circulatory levels of retinol are low in STZ-induced diabetic rats (Basu et al., 1990; Tuitoek et al., 1993), and these levels are not improved with vitamin A supplementation (Tuitoek and Basu, 1994). However, the hepatic levels are increased in ad libitum fed rats and this is magnified with vitamin A supplementation. One of the underlying causes for this increase in liver levels of vitamin A appears to be the increased food intake by the diabetic rats (Table 4-1). Indeed, with pair-feeding, the plasma retinol levels were still reduced by almost 40%, despite unchanged hepatic vitamin A (Table 3-3, chapter 3). These results may also point to the fact that there might be increased absorption and decreased utilization of the vitamin in the presence of diabetes. Since vitamin A is lipid soluble, its absorption may be increased because uptake of lipids is known to be enhanced in STZ-induced diabetic rats (Thomson, 1983a, 1983b). If this is so, it may likely lead to vitamin hepatoxicity.

To the best of our knowledge, the present study is the first report describing vitamin A uptake by the intestine in the presence of diabetes. Retinol was used as a substrate because vitamin A in the diet is present as esters and must be hydrolysed to free retinol before absorption (Fernandez and Borgstrom, 1990). The intestinal uptake of retinol was similar in the control and diabetic rats, indicating that STZ-induced diabetes does not affect the bioavailability (absorption) of retinol. This has been supported indirectly by the fact that when equal amounts of vitamin A were fed to both control and diabetic animals through pair-feeding, the amounts of vitamin A stored in the liver were the same (Chapter 3). The plasma concentrations of the vitamin were however, found to be decreased in the diabetic animals.

The decreased plasma vitamin A in the diabetic rats does not appear to be caused by its impaired intestinal absorption. It is probable that the availability of the vitamin from its hepatic stores is affected by diabetes. This hypothesis can be supported by decreased plasma levels of retinol-binding protein (RBP) in patients with type I diabetes (Basu et al., 1989). Future work should examine if STZ-induced diabetes affects the synthesis of vitamin A carrier proteins, such as RBP and TTR.

The increased hepatic vitamin A concentrations observed in ad libitum fed diabetic rats (Chapter 3) is likely due to the absorption of a normal percentage of an increased dietary intake of vitamin A, rather than due to a primary change in the intestinal permeability to this fat soluble vitamin. The absorption of vitamin A is by passive diffusion, and the uptake of other lipids such as cholesterol and long chain fatty acids is increased in STZ-induced diabetic rats (Thomson, 1980; 1983a). The intestinal absorption of vitamin A is complex, requiring hydrolysis of retinyl ester to free retinol before it is absorbed (Fernandez and Borgstrom, 1990). It is unclear why retinol uptake is unchanged in diabetes.

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5. EFFECT OF STREPTOZOTOCIN-INDUCED DIABETES ON THE RETINOL CARRIER PROTEINS

INTRODUCTION

Vitamin A status is of concern in patients with diabetes mellitus as indicated by a decreased concentration of plasma retinol and its carrier protein, retinol-binding protein (RBP) (Basu et al., 1989; Krempf et al., 1991; Martinoli et al., 1993). A decreased plasma retinol and increased hepatic vitamin A concentrations have been associated with streptozotocin (STZ)-induced diabetic rats (Basu et al., 1990). Vitamin A supplementation to these animals does not improve the circulatory status of retinol while liver concentration further increases (Tuitoek and Basu, 1994). The increased hepatic vitamin A concentrations do not appear to be due to increased intestinal absorption (Chapter 4) but may be attributed in part to the increased food consumption by diabetic rats (Basu et al., 1990). However, the fact that plasma retinol levels are low in diabetic rats, despite the increased hepatic store size of the vitamin, may suggest a defect in mobilization of vitamin A from the liver.

Liver vitamin A is stored predominantly as retinyl esters (Goodman and Blaner, 1984). Its mobilization requires retinyl ester hydrolysis to free retinol. This is subsequently conjugated with RBP (molecular weight of 21,000) synthesized by the liver (Goodman, 1974). The retinol-RBP complex (holo-RBP) is secreted into the circulation, where it binds with transthyretin (TTR), a thyroxine-binding protein (molecular weight of 55,000) (Smith and Goodman, 1979). When retinol is delivered to target cells, RBP loses its affinity for TTR, returns to the blood as apo-RBP (lacking retinol) and is then eliminated via glomerular filtration (Goodman, 1984).

Using STZ-induced diabetic rats, the present study was undertaken to investigate the distributions of RBP and TTR in the liver, kidney and the plasma. The objective of this study was to examine if the reported decreased plasma retinol concentrations in diabetes are caused by a decreased availability of these carrier proteins from the liver or their increased loss from the kidney.

MATERIALS AND METHODS

Animals and tissue collections

Male Wistar rats were obtained from Charles River, Montreal, Canada. They were housed in stainless steel cages in a well-ventilated room maintained at 21^0 C and were on a 12-hour light-dark cycle. Diabetes was induced by single intravenous injection of STZ as indicated in chapter 2. Following injection with STZ, animals displaying plasma glucose of greater than 18 mmol/L were considered diabetic. These diabetic animals and the corresponding controls were fed a semisynthetic diet as shown elsewhere (Table 2-1, chapter 2) for 6 weeks. All animals were allowed free access to water and a record of food intake and body weight was kept throughout the study. Animals were sacrificed at the end of the study using carbon dioxide. Blood was collected in heparinized tubes; separated plasma was protected from light and stored at -20^0 C, pending analysis. Livers and kidneys were removed, cleaned and frozen immediately in liquid nitrogen.

Radioimmunoassays

For RBP and TTR analysis, the liver and kidneys were homogenized separately in 19 volumes (wt/v) of 250 mmol/L sucrose with a Polytron homogenizer at a speed 5 for 15 sec. Aliquots of the homogenates were diluted with an equal volume of a Triton X-100 solution (20 g/L) to release the RBP and TTR trapped in subcellular organelles. The Triton X-100 treated homogenates were diluted with assay buffer (50 mmol/L) Tris-HCl containing 10 g/L bovine serum albumin, pH 8.6), so that the protein concentrations would fall into the most sensitive regions of the respective standard curves. For the determinations of RBP and TTR in plasma, the samples were diluted with assay buffer.

The retinol carrier proteins were measured by radioimmunoassays following the procedures described by Smith et al. (1978; 1980) for RBP and Navab et al. (1977) for TTR. The anti-rat antisera for the transport proteins were prepared at Pennsylvania State university, PA. Purified rat RBP or TTR was iodinated with ^{125}I using a carrier-free $Na^{125}I$. The iodinated protein (RBP or TTR) was separated from free ^{125}I by gel filtration on a column of Sephadex G-50 (Pharmacia Fine Chemicals). The protein was eluted with 0.07 M barbital buffer, pH 8.6; fractions were collected in vials each containing bovine serum albumin which was assayed for ^{125}I. The vial containing the eluted labeled protein was placed on ice, 1 drop of 5% bromphenol blue was added and the solution mixed. Iodinated protein was isolated by vertical gel electrophoresis for 17 hours at 4^0 C in 0.3 M borate buffer, pH 8.6.

A mixture of assay buffer, sample to be assayed, iodinated protein and antirat antiserum for the protein, was incubated in the dark at 4^0 C for 3 days. Each tube was then mixed with goat antirabbit gamma globulin antiserum for RBP and polyethylene glycol for TTR; and incubated at 4^0 C to precipitate the antibody-bound ^{125}I-protein. After incubation, precipitates were collected by centrifugation at 7,000 rpm for 15 minutes at 4^0 C. The supernatant solutions were removed, the precipitates washed with barbital-albumin buffer, and the wash and supernatants combined. The samples were then assayed for ^{125}I. In the assay, ^{125}I found in the precipitate represented antibody-bound RBP-^{125}I or TTR-^{125}I, whereas ^{125}I in the supernatant-wash solution represented free protein. After radioassay, the bound/free ratios of RBP or TTR-^{125}I were calculated for each sample, and the amount of RBP or TTR in the assay tube was then determined from the standard curve. To determine the molar concentrations a molecular weight of 21,422 for RBP, based on the amino acid sequence of rat RBP (Sundelin et al. 1985a), was used, and a molecular weight of 54,792, based on the amino acid sequence of rat TTR (Sundelin et al. 1985b), was used for TTR. Albumin was assayed in the plasma samples by the Bromcresol method (McPherson and Everard, 1972).

Determination of vitamin A

Plasma retinol (Nierenberg and Lester, 1985) and liver total vitamin A (Frolik and Olson, 1984) were assayed by high-performance liquid chromatography as described in Chater 2.

Statistical Analysis

Data were tabulated to give means and standard error of the mean using SAS computer program (SAS Institute, Cary, NC). Student's t-test was used to determine differences between group means (Steel and Torrie, 1980). The upper level of significance was preset at $p < 0.05$.

RESULTS

Following the administration of streptozotocin, animals developed hyperglycemia of more than 18 mmol/L within 48 hours, which persisted throughout the 6 week study period. The initial body weights of diabetic rats were similar to control animals but their final weights at the end of the study were significantly lower ($p < 0.0001$). Thus the diabetic animals gained less weight than did the corresponding controls ($p < 0.0001$), even though they consumed a significantly higher amount of food per day ($p < 0.0001$) as shown in Table 5-1.

TABLE 5-1. Effect of diabetes on animal characteristics (mean ± SEM).

	Control (n = 10)	Diabetic (n = 11)	p-value
Body weights (g):			
Initial	328 ± 4.1	328 ± 6.9	0.98
Final	437 ± 4.7	346 ± 7.1	0.0001
Gain	109 ± 3.1	18 ± 7.6	0.0001
Food intake (g/day)	25.5 ± 0.4	46.2 ± 1.5	0.0001

Significantly lower plasma concentrations of RBP ($p < 0.0001$), TTR ($p<0.0001$) and retinol ($p < 0.05$) were found in diabetic rats compared to controls (Table 5-2). Unlike the retinol carrier proteins, plasma albumin concentrations were not significantly different between the two groups.

TABLE 5-2. Plasma concentrations of retinol, retinol transport proteins and albumin in STZ-induced diabetic rats (mean ± SEM)

	Control (n = 10)	Diabetic (n = 11)	p-value
Retinol (μmol/L)	1.32 ± 0.06	0.90 ± 0.04	0.0001
RBP (μmol/L)	3.72 ± 0.09	2.24 ± 0.17	0.0001
TTR (μmol/L)	3.91 ± 0.12	2.94 ± 0.08	0.0001
Albumin (g/L)	31.9 ± 4.54	25.2 ± 1.17	0.14

Liver concentrations of TTR ($p < 0.0001$) but not RBP ($p > 0.05$) were found to be significantly reduced by diabetes (Table 5-3). However, total (absolute amounts) RBP concentrations in the liver were significantly ($p < 0.01$) lower in the diabetic than in the control animals. Hepatic concentrations of vitamin A were significantly higher in the diabetic than in the control animals ($p < 0.0001$).

TABLE 5-3. Liver concentrations of retinol transport proteins in STZ-induced diabetic rats (mean ± SEM)

	Control (n = 10)	Diabetic (n = 11)	p-value
RBP:			
nmol/g	1.64 ± 0.05	1.48 ± 0.08	0.13
nmol/liver	30.0 ± 1.04	24.5 ± 1.59	0.01
TTR:			
nmol/g	0.92 ± 0.03	0.54 ± 0.02	0.0001
nmol/liver	16.9 ± 0.60	8.84 ± 0.36	0.0001
Vitamin A:			
µmol/g	0.51 ± 0.05	1.03 ± 0.02	0.0001
µmol/liver	9.42 ± 0.36	16.9 ± 0.65	0.0001
Liver weight (g)	18.4 ± 0.36	16.4 ± 0.38	0.002

In the kidney, the tissue concentrations of both RBP ($p < 0.05$) and TTR ($p < 0.0001$) were depressed in the diabetic as compared to the control animals (Table 5-4). However total kidney RBP was not different between the two groups.

TABLE 5-4. Kidney concentrations of retinol transport proteins in STZ-induced diabetic rats (mean ± SEM)

	Control (n = 10)	Diabetic (n = 11)	p-value
RBP:			
nmol/g	4.48 ± 0.07	4.13 ± 0.12	0.02
nmol/kidney	8.32 ± 0.27	8.86 ± 0.29	0.19
TTR:			
nmol/g	0.79 ± 0.03	0.56 ± 0.02	0.0001
nmol/kidney	1.46 ± 0.06	1.21 ± 0.05	0.006
Kidney weights (g)	1.84 ± 0.04	2.15 ± 0.04	0.0001

DISCUSSION

The results of the present study indicate that plasma retinol concentrations are significantly lower, and the hepatic concentrations of vitamin A are significantly higher in the diabetic verses control animals. These results are in agreement with other studies in humans (Basu et al. 1989; Krempf et al. 1991; Martinoli et al. 1993) and animals (Basu et al. 1990, Tuitoek and Basu, 1994). Since it has been shown that the hepatic storage of vitamin A is proportional to its intake (Hicks et al., 1984), the increased liver concentrations of vitamin A may be explained in part by the increased food intake (> 45%) of the diabetic animals.

The present study also examined the effect of diabetes on RBP and TTR in the plasma, liver and kidneys. The results show that there were significant reductions in concentrations of both of these proteins in the plasma and kidneys. These effects appear to be specific since the plasma albumin concentrations were unaffected. To the best of our knowledge, this is the only study where plasma retinol carrier proteins have been measured in diabetic rats. The results are in agreement with a human study in which plasma RBP concentration was found to be significantly reduced in IDDM patients, paralleling the reduced levels of plasma retinol (Basu et al., 1989). The underlying reason for this reduced plasma RBP and TTR in diabetes is not known. Several clinical and nutritional deficiency conditions including vitamin A and zinc deficiencies, liver diseases and protein calorie malnutrition (PEM) have revealed similar reductions of plasma retinol, RBP and TTR and may explain in part the results of our study (Smith and Goodman, 1971; Smith et al., 1974; Shetty et al., 1979; Polberger et al., 1990).

Secretion of RBP from the hepatocyte is strictly regulated by its ligand, retinol. In vitamin A deficiency, hepatic secretion of RBP is specifically inhibited so that its plasma concentration falls and apo-RBP accumulates in the liver (Muto & Goodman, 1972). Upon vitamin A repletion to deficient rats, RBP is rapidly secreted into the plasma (Smith et al., 1973). The retinol-induced secretion of RBP has been shown to be independent of protein synthesis (Soprano et al., 1982). In the present study, while plasma RBP concentration was reduced, there was no increase in its hepatic concentrations, suggesting that the depressed plasma RBP was not due to depleted hepatic levels of vitamin A. Furthermore earlier studies have shown that the diabetes-associated increase in hepatic vitamin A concentration (Basu et al., 1990) is further accentuated when vitamin A is supplemented in the diet without a significant improvement in the decreased plasma retinol (Tuitoek and Basu, 1994).

Acute and chronic diseases of the liver have been shown to be associated with decreased plasma vitamin A, RBP and TTR (Smith and Goodman, 1971; McClain et al., 1979). These low levels of RBP and TTR presumably reflect a reduced rate of production of the proteins by the diseased liver (Goodman, 1984). However, in situations such as in alcoholic cirrhosis where reduced plasma vitamin A, RBP and TTR are accompanied by poor zinc status, vitamin A supplementation alone did not improve the abnormal dark adaptation suffered by the patients (Morrison et al., 1978). However, normalization was accomplished when vitamin A was supplemented with zinc, suggesting that these patients had inability to mobilize vitamin A from the liver. In STZ-induced diabetes, hepatic vitamin A is increased while plasma retinol levels are reduced suggesting that mobilization of the vitamin from the liver is impaired. Furthermore, zinc metabolism is known to be affected in diabetes as evident by hyperzincuria (Kinlaw et al., 1983, Lau and Failla, 1984). Future studies should examine whether zinc supplementation has the possibility of mobilizing vitamin A from the liver to plasma.

Smith et al. (1974) have also shown that rats on a zinc deficient diet have significantly reduced plasma vitamin A, RBP and hepatic RBP. It has been suggested that the reduced plasma RBP is caused by the depressed synthesis of hepatic RBP (Smith et al., 1974). The lower level of plasma RBP was much more pronounced than the general decrease in plasma protein observed in zinc-deficient rats, suggesting a specific effect on RBP. In protein-energy malnutrition (PEM), plasma RBP, TTR and vitamin A have all been found to be reduced, possibly due to impaired hepatic synthesis of the vitamin A carrier proteins caused by inadequate intakes of vitamin A and zinc. Since both RBP and TTR are rapidly turning over (half-lives, 12 hours and 2-3 days, respectively) plasma proteins, it can be anticipated that their plasma levels might provide a sensitive index for mild degrees of PEM as compared to plasma levels of more slowly turning over proteins such as albumin (half-life, 14 days) (Goodman, 1984). Thus many studies have suggested that plasma RBP and TTR are useful indicators for assessing early PEM (Shetty et al. 1979; Wade et al. 1988; Polberger et al., 1990). Polberger et al. (1990) have found that protein energy rather than total energy intake appear to provide the main contribution to the changes found in plasma RBP and TTR in preterm infants. In obese women, while plasma albumin and transferrin failed to respond to short-term nutritional changes, levels of plasma TTR and RBP were found to be sensitive indicators of early PEM (Shetty et al., 1979). Since plasma albumin was not affected by diabetes in the present study, the decreased levels of RBP and TTR in the plasma and liver may be an early indication of depressed protein synthesis. Furthermore STZ-induced diabetes has been shown to be associated with depressed synthesis of both secreted and nonsecreted proteins in the liver (McNurlan and Garlick 1981). It has also been found in diabetic animals that although hepatic synthesis of some proteins such as albumin appear reduced as shown by decreased mRNA levels (Peavy et al. 1978), the serum albumin levels remain normal (Marsh, 1961). In the present study, hepatic RBP concentrations were not as affected as TTR concentrations, suggesting that TTR may be a more sensitive index of impaired protein synthesis in diabetes or more sensitive to insulin lack. The formation of the RBP-TTR complex is thought to occur in plasma after independent secretion of the proteins from the hepatocyte (Goodman, 1984). The reduced secretion of TTR may decrease the formation of the RBP-TTR complex, resulting in an increased loss of RBP from the system. Since the plasma TTR molar concentrations in the diabetic rats was 1.3 times the plasma molar RBP concentration, RBP is probably not being filtered in the kidney glomerulus at a greater rate because of the lower TTR concentrations.

Once RBP has given up retinol in the target tissues, the resulting apoRBP has a reduced affinity for TTR. Due to its low molecular weight, the apoRBP rapidly undergoes glomerular filtration in the kidney (Peterson et al., 1974). Like other small proteins that undergo glomerular filtration the RBP is reabsorbed in the kidney tubules and seems to be catabolized (Mogielnicki et al., 1971). Only trace amounts of RBP appear in the urine unless kidney tubular function is impaired (Peterson and Berggård, 1971). In conditions of impaired tubular fuction, such as cadmium poisoning, an amount of RBP equivalent to the amount secreted into the plasma compartment can be excreted in the urine each day. However, these patients maintain normal plasma concentrations of RBP because the impaired tubular reabsorption does not influence the glomerular filtration rate (Vahlquist et al., 1973). IDDM patients have increased urinary excretion of RBP (Rowe et al., 1987, Holm et al., 1987). Since urinary excretion of RBP was not accompanied by the excretion of albumin, the patients seem to have normal glomerular function but have impaired tubular function. The urinary excretion of RBP was suggested to be an early sign of diabetic nephropathy. The low concentration of RBP in the kidneys of the diabetic rats probably reflects (i) an impaired tubular function that allows much of the RBP to appear in the urine and (ii) a reduced secretion of RBP into the plasma; therefore, less RBP is available to be removed by the kidneys.

The conclusion from this study is that STZ-induced diabetes is associated with depressed plasma retinol concentration which may be due, at least in part, to impaired availability of its plasma carrier proteins.

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6. EFFECT OF INSULIN TREATMENT OR ZINC SUPPLEMENTATION ON VITAMIN A STATUS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

INTRODUCTION

Vitamin A metabolism has been shown to be abnormal in diabetes mellitus as indicated by decreased levels of plasma retinol and retinol-binding protein (RBP) in patients with insulin-dependent diabetes (IDDM) (Basu et al., 1989; Krempf et al. 1991; Martinoli et al., 1993). It has further been shown that diabetic patients not only have reduced plasma retinol but also elevated levels of retinyl esters (Wako et al., 1986), reflecting an increased hepatic storage. Streptozotocin (STZ)-induced diabetic rats have decreased plasma retinol levels which are accompanied by an elevated hepatic concentration of vitamin A (Basu et al., 1990). The decreased plasma level of the vitamin remaines unchanged in the diabetic animals even when they consume a diet supplemented with 10 times the basal level of vitamin A while their hepatic levels of vitamin A accentuated (Chapter 3). The reason for the increased storage of vitamin A in the liver is not well understood but has been attributed in part to increased food intake by the diabetic animals. An increased intestinal absorption does not appear to contribute to the increased hepatic vitamin A (Chapter 4). The mobilization of retinol from the liver requires RBP which binds with retinol for secretion into the plasma where retinol-RBP complex further binds with TTR before being transported to the target tissues. These carrier proteins have been found to be decreased in the plasma, liver as well as in the kidney of STZ-induced diabetic rats (Chapter 5), suggesting an impaired mobilization of vitamin A from its storage to the circulation.

Zinc is required for the synthesis of RBP (Smith et al., 1974; Smith, 1982) and has been shown to promote the transport of vitamin A from the liver (Morrison et al., 1978; Ette et al., 1979). Since zinc metabolism has been known to be perturbed in the presence of diabetes (Kinlaw et al., 1983; Lau and Faila, 1984; Walter et al., 1991; Uri-Hare et al., 1992), it is possible that zinc supplementation may enhance the hepatic synthesis of RBP and thereby reverse the decreased plasma retinol found in diabetes.

While the reason for the decreased plasma retinol levels has not been fully understood, it has been demonstrated that administration of insulin to non-diabetic rats results in an increased depletion of vitamin A stores from the liver (Bowles, 1967). Further, administration of insulin to diabetic rats has been shown to normalize plasma retinol but not liver vitamin A concentrations (Leichter et al., 1991). However, insulin administration to the diabetic rats was started 3 weeks after diabetes was induced and animals were injected with one dose of insulin daily.

Using STZ-induced diabetic rats, the present study was undertaken to investigate the effect of zinc supplementation in the diet and subcutaneously implanted insulin pellets on plasma and liver vitamin A and zinc levels. The objective being to examine if reduced plasma levels of retinol would be reversed.

MATERIALS AND METHODS

Animals and diet

Male Wistar rats (Crl: (WI)BR), weighing 225-250 g were obtained from Charles River, Montreal, Canada. They were housed in stainless steel cages in a well-ventilated room maintained at 21^0 C and were on a 12-hour light-dark cycle. Diabetes was induced by a single intravenous injection of STZ as described in chapter 2. Following injection with STZ, animals displaying plasma glucose of greater than 18 mmol/L were considered diabetic.

Animals were divided into four groups: Group A was the non-diabetic control, and the remaining groups were all diabetics with (group B) and without (group C) zinc supplementation or treated with insulin (group D). All animals were allowed free access to a semisynthetic diet described elsewhere (Chapter 2) for 4 weeks. Group C were fed the semisynthetic diet supplemented with zinc sulfate (120 µg/g diet).

Insulin treatment

Group D was treated with insulin through implantation. The 7-mm long and 2 mm diameter implants, made by high pressure compression of a powder admixure of insulin and re-crystallized palmitic acid were obtained from Institute of Biomedical Engineering, University of Toronto, Canada. Unlike insulin given by injection, the implant releases a set basal dose of insulin continuously throughout the day. The rats in this study were less than 300 g in body weight and each received 1 full-size (7 mm long) and 1 half-size pieces of implants as recommended by Wang (1991).

The animals were anesthetized with halothane, the neck region was shaved, and the the implant was inserted. After cleaning the shaved spot with betadine, the skin was pinched between the thumb and index fingers. The skin was then pierced with a 16G disposable hypodermic needle and withdrawn. The trocar was immersed briefly in 2% betadine solution, then it was pushed through the skin orifice just created to a length of at least 2 cm. The insulin implant was immersed briefly on the 2% betadine solution and then inserted into the proximal end of the trocar. The obturator was then used to push the implant until it exited from the distal end of the trocar. The skin was pinched over the inserted implant, before withdrawing the trocar. The skin defect was closed with one metal clip.

A record of food intake and body weight was kept throughout the study. Animals were sacrificed at the end of the study using a carbon dioxide. Blood was collected in heparinized tubes; separated plasma was protected from light and stored at -20^0 C, pending analysis. Livers were removed, cleaned and frozen immediately in liquid nitrogen.

Determination of vitamin A

Plasma (Nierenberg and Lester, 1985) and liver (Frolik and Olson, 1984) vitamin A was assayed by high-performance liquid chromatography as described in chapter 2.

Zinc determinations

Zinc content of tissues was analyzed using the atomic absorption spectrophotometry (Perkin Elmer Model) with a zinc standard (Aldrich Chemical Co.). All glassware was rinsed in diluted nitric acid and deionized water to avoid any contamination (Liska et al., 1985). Standards were prepared by diluting the stock standard solutions with saline.

Plasma was diluted 1/10 with deionized water. Liver samples were analyzed for zinc by modification of the method used by Oster et al. (1989). Weighed liver samples were dried in preweighed porcelain crucibles for 23 hrs at 110^0 C and cooled in a dessicator. Concentrated sulphuric acid (H_2SO_4) was added and the samples were digested on a block heater for 40 minutes. A mixture of concentrated nitric acid/perchloric acid (72%)/ H_2SO_4; by volume 8:2:3, was added and digestion continued for a further 30 minutes, until a clear solution was produced. The cooled solution was transferred quantitatively to a volumetric flask, diluted with deionized water and mixed for analysis.

Statistical Analysis

Data were tabulated to give means and standard errors of the mean using SAS computer program (SAS Institute, Cary, NC). The four treatments were compared using one way analysis of variance (ANOVA) (Steel and Torrie, 1980). When significant differences were detected by ANOVA, the appropriate comparisons were made. The upper level of significance was preset at $p < 0.05$.

RESULTS

As expected all the diabetic animals exhibited elevated plasma glucose (> 18 mmol/L) levels within 48 hours of streptozotocin injection. The untreated diabetic rats exhibited a reduction in body weight gain, while their daily food intake was approximately 50% more than the controls (Table 6-1). In diabetic animals treated with insulin for 4 weeks, the plasma glucose, body weight gain and daily food intake were at non-diabetic control levels, indicating treatment of diabetes. However, this was not the case with diabetic animals fed a zinc supplemented diet. These animals displayed mean body weight gain, daily food intakes and plasma glucose levels which were similar to those of the untreated diabetic rats.

TABLE 6-1. Effect of insulin treatment and zinc supplementation on animal characteristics[1]

	Body weight gain (g)	Mean daily food intake (g)	Plasma glucose (mmol/L)	Liver wt (g)
Control	154 ± 10.3^{a}	21.8 ± 2.10^{a}	6.9 ± 2.03^{a}	14.2 ± 0.7^{a}
Diabetic	48 ± 9.5^{b}	42.3 ± 1.95^{b}	20.7 ± 1.54^{b}	12.7 ± 0.6^{a}
Diabetic, zinc supplemented	47 ± 10.3^{b}	44.5 ± 2.10^{b}	20.6 ± 1.66^{b}	12.9 ± 0.7^{a}
Diabetic, insulin treated	144 ± 9.5^{a}	22.1 ± 1.95^{a}	8.2 ± 1.82^{a}	12.1 ± 0.6^{a}

[1] Results are expressed as mean ± SEM at least five animals. a,b letters not shared show significance ($p < 0.05$) as analysed by Scheffe's test.

The differences in body weights and daily food intake over a period of 4 weeks are shown in in Figures 6-1 and 6-2, respectively. Restricted weight gain and increased food intake were evident from the onset of STZ-induced diabetes, and so was the effect of insulin in reversing these characteristics to non-diabetic control levels.

The plasma and liver vitamin A concentrations are shown in Table 6-2. Plasma retinol concentrations were significantly reduced while total hepatic vitamin A was elevated in the diabetic when compared to control rats. When diabetic animals were treated with insulin, the vitamin A status in both plasma and the liver was reversed to control levels. Zinc supplementation, however, failed to show this effect. Thus the diabetic rats fed a diet containing zinc, 120 μg/g for 4 weeks had circulatory and hepatic concentrations of vitamin A similar to that of untreated diabetic animals.

TABLE 6-2. Effect of insulin treatment and zinc supplementation on plasma and liver vitamin A concentrations in STZ-induced diabetic rats[1]

	Plasma retinol (μmol/L)	Liver vitamin A (μmol/g)
Control	1.25 ± 0.03[a]	0.41 ± 0.06[a]
Diabetic	0.95 ± 0.04[b]	1.08 ± 0.07[b]
Diabetic, zinc supplemented	0.96 ± 0.04[b]	1.06 ± 0.07[b]
Diabetic, insulin treated	1.33 ± 0.03[a]	0.43 ± 0.07[a]

[1] Results are expressed as mean ± SEM at least five animals. a,b letters not shared show significance ($p < 0.05$) as analysed by Scheffe.

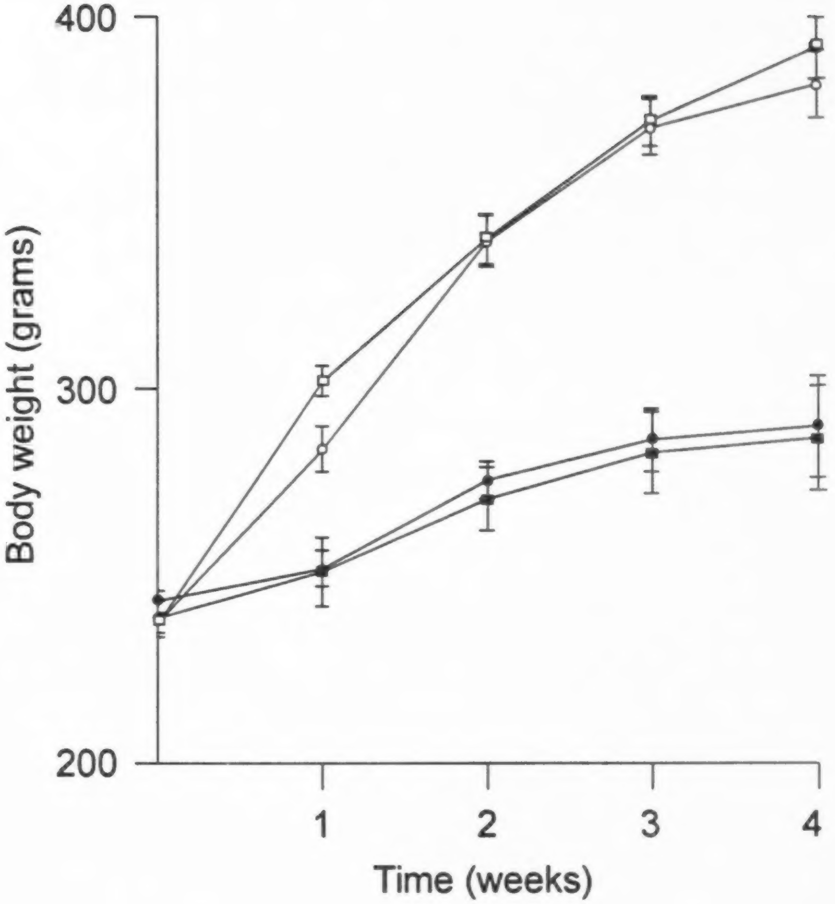

FIGURE 6-1. Mean body weights of control, -□- ; diabetic,-■- ; zinc supplemented diabetic, -●-;and insulin treated diabetic,-○-rats. Each point on the graph represents mean ±SEM of at least 5 animals.

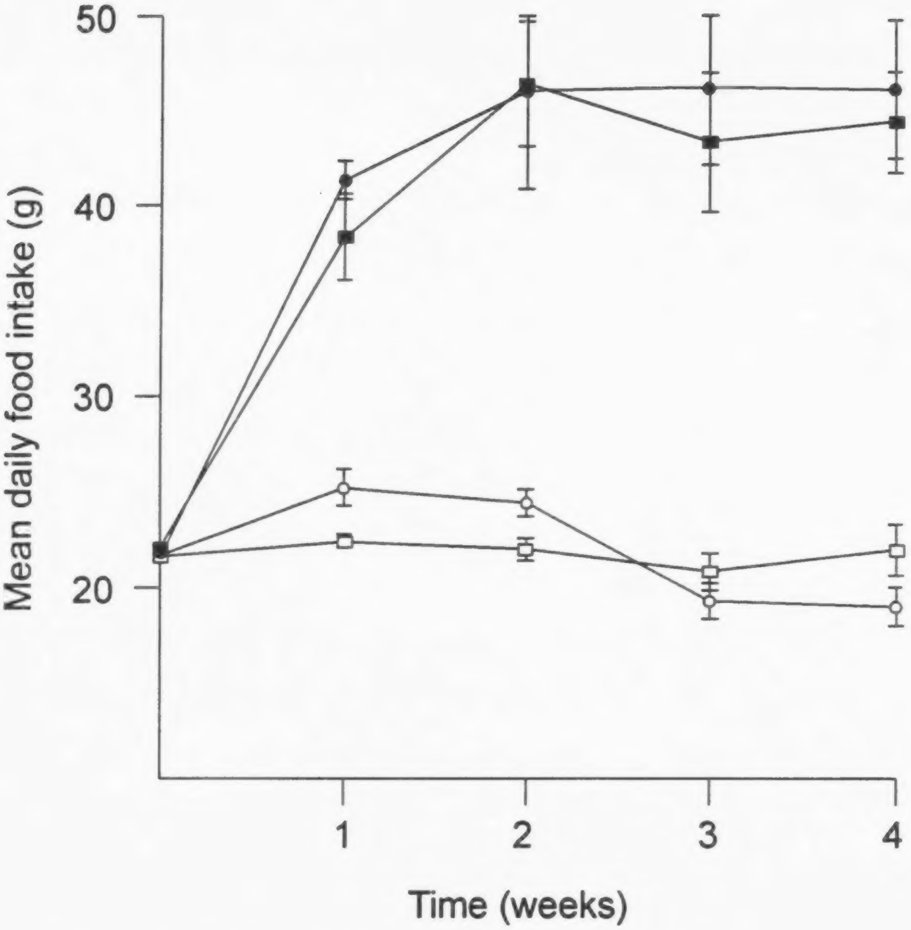

FIGURE 6-2. Mean daily food intake of control, -□-; diabetic,-■- ; zinc supplemented diabetic, -●- ;and insulin treated diabetic,-○-rats. Each point on the graph represents mean ± SEM of at least 5 animals.

The zinc concentrations in the plasma and liver are shown in Table 6-3. Plasma zinc concentrations were not affected by diabetes, and remained unaffected by either zinc supplementation or insulin treatment. However, the liver concentrations of zinc were 16% and 22% higher in diabetic and zinc supplemented diabetic groups, respectively, than the non-diabetic animals.

TABLE 6-3. Effect of insulin treatment and zinc supplementation on plasma and liver zinc levels in STZ-induced diabetic rats[1]

	Plasma zinc (μmol/L)	Liver zinc (μmol/g dry wt)
Control	18.9 ± 1.35	1.93 ± 0.06[a]
Diabetic	18.4 ± 1.25	2.24 ± 0.06[b]
Diabetic, zinc supplemented	18.4 ± 1.35	2.36 ± 0.06[b]
Diabetic, insulin treated	17.4 ± 1.47	2.02 ± 0.06[a]

[1] Results are expressed as mean ± SEM at least five animals. a,b letters not shared show significance ($p < 0.05$) as analysed by Student Newman Keuls test.

DISCUSSION

The results of the present study show that plasma retinol levels were significantly reduced, while the hepatic levels of vitamin A were significantly increased in the diabetic than in the control animals. These results are in agreement with earlier studies involving humans or diabetic rats (Basu et al., 1989; Tuitoek and

Basu, 1994). The increased liver concentrations of vitamin A may be explained in part by the increased food intake by the diabetic animals (> 50%), since it has been shown that the hepatic storage of vitamin A is proportional to its intake (Hicks et al., 1984).

Insulin treatment to the diabetic rats in this study was found to restore plasma retinol levels to the control values. This is in agreement with a previous study in which plasma retinol was restored to normal levels in diabetic rats treated with a single dose of insulin once a day for 3 weeks (Leichter et al., 1991). In that study however, the insulin treatment failed to alter the diabetes associated elevated hepatic vitamin A concentrations. This is not the case with the present study where insulin treatment not only restored plasma retinol but also reduced the liver concentration of vitamin A to its control value. The difference between the results of the two studies might be explained by the time at which insulin treatment was started and the mode of insulin treatment. In the present study, the insulin treatment was started at the onset of diabetes while in the previous study, treatement began 3 weeks after the onset of diabetes. Thus it is possible that the diabetic animals were able to store large quantities of vitamin A in the liver within the 3 weeks before they were treated with insulin. In the previous study insulin treatment once a day did not appear to cause much reduction in the mean daily food intake when compared to the untreated diabetic group. The insulin treated animals had a mean daily food intake 37.7% while the untreated diabetic group 46.9% more than that of the non-diabetic controls. Therefore this increased food intake to the insulin treated diabetic animals may have contributed in part to the increased hepatic storage of vitamin A. Insulin treatment to nondiabetic rats has been shown to increase the rate of depletion of vitamin A from the liver (Bowles, 1967). The results of the present study appear to support this finding because hepatic concentration of vitamin A of the insulin treated group was significantly lower than the untreated diabetic group and was not different from that of the control group.

Since STZ is hepatoxic (Chang, 1981), it is possible that it may have an effect on the vitamin A release from the liver. However, the effect of insulin in restoring plasma vitamin A to normal levels suggests that the defect in the vitamin A metabolism of STZ-induced diabetic rats was primarily due to the insulin-deficient state of the animals, rather than to a direct hepatoxic effect of STZ. Moreover, plasma vitamin A has been found to be lower in IDDM patients than in non-diabetic subjects (Basu et al., 1989).

The decreased plasma concentrations of retinol have been suggested to be due to a deficiency of zinc which is required for the synthesis of the RBP, the protein that binds retinol in the liver for secretion into the plasma (Smith, 1982). Thus in situations such as in alcoholic cirrhosis where reduced plasma vitamin A is accompanied by a poor zinc status, vitamin A supplementation alone did not improve the abnormal dark adaptation suffered by the patients (Morrison et al., 1978). However, normalization was accomplished when vitamin A was supplemented with zinc, suggesting that these patients had inability to mobilize vitamin A from the liver. In diabetes, hepatic vitamin A is increased while plasma retinol levels are reduced suggesting that mobilization from the liver is impaired. Thus in the present study zinc was supplemented in order to examine whether zinc has the possibility of mobilizing vitamin A from the liver to plasma. Furthermore, zinc metabolism is known to be perturbed in the presence of diabetes as evident by hyperzincuria, (Kinlaw et al., 1983), decreased plasma and skeleatal muscle (Sjogren et al., 1988), and lower concentrations in lymphocytes, granulocytes and plateletes (Pai and Prasad, 1988). There was no effect of zinc supplementation on plasma retinol or liver vitamin A levels in the present study. The reason for the increased hepatic vitamin A while plasma levels are reduced is not fully understood but has been suggested to be due in part to reduced hepatic synthesis of its carrier proteins (Chapter 5).

The present study also measured plasma and liver zinc concentrations. While there were no marked changes in the plasma in all the groups of rats, hepatic zinc concentrations of diabetic and zinc supplemented diabetic rats were found to be raised. The present study confirms the findings by others who found increased hepatic zinc levels in diabetic animals (Failla and Kiser, 1981; Raz and Havivi, 1988; Uri-Hare et al., 1992). The fact that there were no differences in hepatic zinc levels between the zinc supplemented and the unsupplemented diabetic rats suggest that the observed changes are due to the insulin deficiency. This is supported by the normalization of hepatic zinc levels in insulin treatment. The accumulation of zinc in the diabetic liver has been shown to parallel increased levels of metallothionein, a zinc-binding cytoplasmic protein (Failla and Kiser, 1983; Uri-Hare et al., 1992). Hormones such as glucagon, glucorcorticoid and epinephrine are known to be stimulators of hepatic metalothionein synthesis as shown by the increased number of its mRNA (Cousins et al., 1986). Thus it has been suggested that insulin deficiency results in the increase of these hormones leading to an increase in hepatic metalothionein and zinc levels (Failla and Kiser, 1983).

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7. GENERAL DISCUSSION AND CONCLUSIONS

Diabetes is a disease of impaired glucose homeostasis where plasma glucose is elevated but it is not utilized adequately by the body. Vitamin A status is also abnormal in diabetes where despite its increased hepatic load, its concentration in circulation and retina of the eye is depressed. However, reasons for impaired homeostasis in glucose and vitamin A in diabetes are different. Abnormality in glucose metabolism in diabetes is due to insulin lack or decreased insulin production or insulin resistance. This causes an increased glycogen, protein and lipid catabolism beyond the fuel needs of the body. Furthermore, the ability of peripheral tissues to utilize glucose is impaired and large quantities are found in circulation as well as lost in the urine. The cause for the impaired metabolic availability of vitamin A in diabetes is not fully understood. Possible factors may include alteration in its absorption and excretion, mobilization from its hepatic storage, hepatic retinyl ester hydrolase activity, synthesis of plasma carrier proteins, zinc metabolism and renal clearance of RBP. Some of these factors have been examined in the present study.

Absorption of lipids is known to be enhanced in the presence of diabetes (Thomson, 1980). Since vitamin A is lipid soluble, it was hypothesized that vitamin A absorption would likewise be enhanced. The uptake of [^{3}H]retinol was examined in jejunum and ileum. The tissues were either incubated for 6 minutes at a concentration of 1-24 μM or at a concentration of 24 μM, for up to 15 minutes. The uptake of retinol was not different between control and diabetic animals at different concentrations or incubation times (Chapter 4). Since vitamin A is present in the diet as retinyl ester and requires the hydrolysis to retinol in the lumen of the gut before absorption, it is important that absorption of vitamin A is carried out in vivo using retinyl ester. It was, however noteworthy that when the same amount of food containing an equal amount of vitamin A was fed to age and weight-matched pairs of control and diabetic rats, no differences in the amount of vitamin A stored in the liver was observed. This finding indicates that it is unlikely that the absorption of vitamin A is affected by diabetes.

Plasma retinol levels are regulated homeostatically by the release from its hepatic stores so that it is independent of short-term fluctuations in dietary intake. Except in extreme deficiency, levels of plasma retinol are not a good indicator of the vitamin A status (Olson, 1991). However, factors other than deficiency can alter the homeostatic regulation of plasma retinol. In certain situations such as in diseases of the liver, stress, protein-energy malnutrition and zinc deficiency, plasma retinol levels are low but unlike in vitamin A deficiency, it appears that there is adequate hepatic storage of vitamin A. It has therefore been suggested that the reduced plasma retinol levels in these situations are due to impaired mobilization of vitamin A from the liver to the plasma. Furthermore, plasma RBP levels in the above mentined situations are lower than in controls, suggesting that synthesis or secretion of RBP may be impaired. Indeed, vitamin A supplementation has not always improved the low plasma vitamin A levels. For example, in PEM where liver biopsy samples showed sufficient storage of vitamin A, feeding of high protein foods to the children without vitamin A supplementation increased their plasma vitamin A and RBP levels. This implied that with adequate protein intake, RBP is synthesized in order to mobilize retinol from the liver to the plasma (Arroyave et al., 1961). In alcoholic cirrhosis, where reduced plasma vitamin A was accompanied by poor zinc status, vitamin A supplementation alone did not improve the abnormal dark adaptation suffered by these patients (Morrison et al., 1978). However, normalization of vision was accomplished when vitamin A was supplemented with zinc, suggesting that vitamin A deficiency in these patients was secondary to zinc deficiency.

Supplementation of the diet with vitamin A did not result in any significant increase in the plasma retinol of diabetic animals, despite its markedly increased hepatic store size (Chapter 3). The increased hepatic storage of vitamin A was attributed in part to the increased intake of vitamin A because the diabetic animals consumed approximately 45% more food than the controls. This is supported by Hicks et al. (1984), who showed that the amount of vitamin A intake is proportional to hepatic storage of vitamin A. This was further supported by pair-feeding where intake of the same amount of food containing approximately equal amounts of vitamin A, resulted in no difference in liver total vitamin A between the control and the diabetic rats. Thus hepatic vitamin A in ad libitum fed diabetic rats may be due to absorption of a normal percentage of an increased dietary intake of vitamin A, rather than due to a primary change in the intestinal permeability to vitamin A. The fact that there was increased vitamin A in the hepatic storage and decreased concentrations in circulation suggests impaired mobilization of vitamin A.

Vitamin A is stored as retinyl ester but circulates in the plasma as free retinol bound to RBP and TTR. Thus, before secretion from the liver into the plasma, retinyl ester is hydrolyzed to retinol by retinyl ester hydrolase enzyme. It is possible that this enzyme activity in diabetes may be reduced since there is increased hepatic total vitamin A while its circulatory levels are low. However, the free retinol concentrations in the liver of the diabetic rats were found to be significantly higher than in the pair-fed controls. Therefore this argues against any decreased activity of the enzyme. The increased hepatic free retinol levels accompanied by depressed circulatory levels in the diabetic animals however, suggest that there may be an impaired mobilization of vitamin A from its hepatic stores into circulation. In zinc deficiency, plasma retinol is low while the hepatic vitamin A is normal (Smith et al., 1974), indicating that its mobilization is reduced. The reduced plasma vitamin A in zinc deficiency has been suggested to be due to reduced RBP synthesis because both hepatic and circulatory RBP were low.

Further examination of the possibility that reduced plasma retinol concentration is due to changes in concentrations of carrier proteins, RBP and TTR in the plasma, liver and kidney was studied. These proteins are synthesized in the liver and secreted to the plasma (Chapter 5) . The secretion of RBP but not of TTR from the liver is dependent on the availability of vitamin A (Navab et al., 1977). In vitamin A deficiency, RBP levels in the liver are elevated while its plasma concentrations are low (Soprano et al., 1982). When vitamin A is repleted to vitamin A deficient rats, the liver RBP levels gradually decrease while plasma levels increase. However, RBP synthesis is not dependent on vitamin A availability because despite the use of protein inhibitors before repletion with vitamin A to deficient rats, plasma RBP levels increase, suggesting an effect on hepatic secretion of RBP and not synthesis (Smith et al., 1973). In the present study, plasma RBP and TTR levels were found to be lower in the diabetic than in control rats. The reasons for this decrease are not known but plasma RBP and TTR have been shown to be sensitive to disease (Smith and Goodman, 1971), inadequate intake of protein (Polberger et al., 1990) and stress (Cynober et al., 1985).

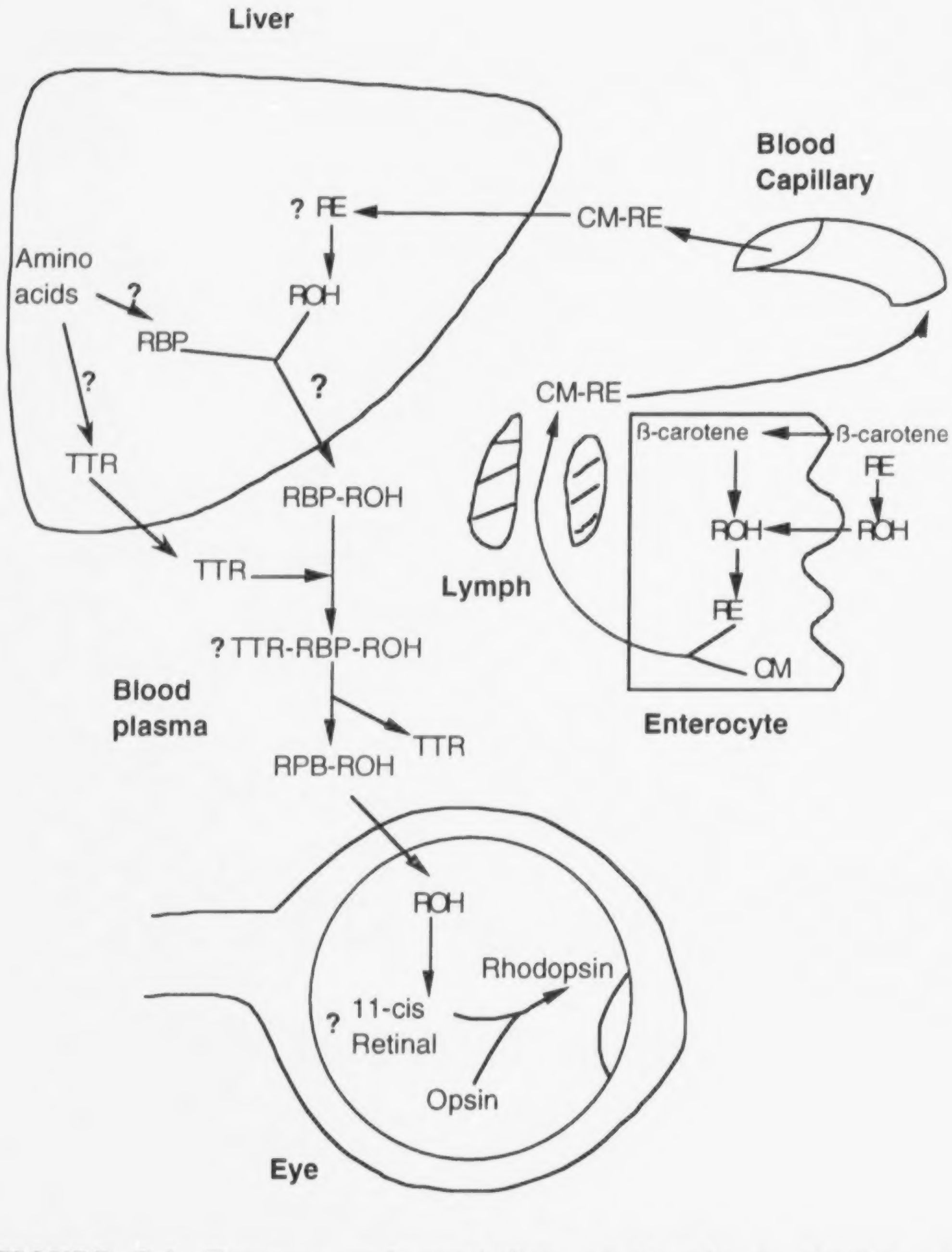
In patients with acute and chronic diseases of the liver, plasma RBP and TTR are low, presumably due to their decreased synthesis by the malfunctioned liver (Smith and Goodman, 1971). It may be argued that the streptozotocin was used to induce diabetes in the present study, and that this agent is known to be hepatoxic (Chang, 1981). The reduced synthesis of the retinol carrier proteins may be a reflection of the hepatoxic effect. However, the fact that insulin administration to these STZ-induced diabetic rats restored the plasma and liver vitamin A concentrations to normal reduces this possibility.

Plasma RBP and TTR are known to be sensitive to changes in protein status (Wade et al., 1988; Polberger et al., 1990). No evidence of changes in the plasma albumin, an indicator of protein status, was found in STZ-induced diabetic rats. However, synthesis of albumin has been shown to be impaired in STZ-induced diabetic rats even though its serum levels remain normal (Peavy et al., 1978; Marsh, 1961). It is therefore possible that the reduced plasma RBP and TTR levels in STZ-induced diabetic rats are an early reflection of protein malnutrition. Moreover, insulin deficiency is known to alter protein metabolism. Insulin is required for uptake of amino acids and to stimulate protein synthesis and inhibit protein degradation. Thus in insulin deficiency, storage of protein is reduced and protein degradation is increased (Nair et al., 1983; Luzi et al., 1990).

Stress of various kinds such as physical immobilization, fever, burn injury and chronic infections, appear to lower circulatory retinol and enhance vitamin A catabolism (Morita and Nakano, 1982; Cynober et al., 1985; Campos et al., 1987; Takase et al., 1992). Factors involved in stress-related decrease of plasma vitamin A are not fully understood. However, stress situations are known to trigger secretion of hormones and have been shown to cause depletion of vitamin A from plasma, thymus and liver (Atukorala et al., 1981). Insulin deficiency is accompanied by an increase in stress-related hormones in the body. In the present study however, mobilization of vitamin A from the liver was reduced rather than enhanced as seen in the stress-related situations; thus in the present study changes in vitamin A metabolism may not be stress hormone-associated.

Furthermore, insulin treatment to normal rats causes the depletion of vitamin A from the liver (Bowles, 1967). Also insulin administration to diabetic rats in the present study showed a normalization of vitamin A in the plasma and liver (Chapter 6), indicating the importance of insulin or control of hyperglycemia in the metabolism of vitamin A. Figure 7-1 shows some of the steps (indicated by question marks) which are suggested to be interrupted in the presence of diabetes. These include the inadequate synthesis of RBP and TTR in the liver, thus reduced formation of TTR-RBP-retinol complex into the plasma, hence reduced contents of cis-retinal in the retina of the eye.

Since zinc is necessary for the synthesis of RBP, it was hypothesized that supplementation with zinc in the diet would increase the circulatory vitamin A concentrations. Previous reports indicate the beneficial effects of zinc supplementation on vitamin A metabolism in malnourished children (Shingwekar et al., 1979), adults with alcoholic cirrhosis (Morrison et al., 1978) and preterm infants (Hustead et al., 1988). In these situations however, reduced levels of plasma vitamin A are accompanied poor zinc status. Zinc metabolism has been shown to be disturbed in the presence of diabetes as indicated by its increased urinary losses (Kinlaw et al., 1983) and reduced tissue levels (Pai and Prasad, 1988). While plasma levels were unaffected, liver concentrations of zinc were higher in the diabetic rats as compared to controls in the present study, indicating that its metabolism maybe impaired. In the present study, STZ-induced diabetic rats were supplemented with


Liver
Blood
Capillary
? RE
CM-RE
Amino
acids
?
ROH
RBP
?
?
TTR
RBP-ROH
CM-RE
ß-carotene
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ROH
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Lymph
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? TTR-RBP-ROH
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Blood
plasma
Enterocyte
TTR
RPB-ROH
ROH
Rhodopsin
?
11-cis
Retinal
Opsin
Eye

FIGURE 7-1. Transport and metabolism of vitamin A, showing the steps which maybe interrupted in the presence of diabetes. ROH, retinol; RE, retinyl esters; CM, chylomicrons; RBP, retinol binding protein; TTR, transthyretin.

zinc, 10 times the amount in the basal diet. However, zinc supplementation did not have any effect on vitamin A status in diabetic rats. It is possible that the duration of diabetes in the present study was not long enough to cause significant changes in zinc metabolism. However these findings do not preclude zinc as an additional factor in vitamin A metabolism in diabetes.

Results from the present study suggest that vitamin A metabolism in diabetes is impaired as shown by its decrease in circulation and target tissue, the retina, while its hepatic storage is increased. However, in vitro intestinal absorption of retinol was not altered by diabetes. Supplementation of vitamin A did not have a significant improvement in the circulatory vitamin A. It is important to note that vitamin A supplementation increased further its hepatic storage which if continued may lead to hepatoxicity (Bendich and Langseth, 1989). Although no direct measurement of synthesis of retinol carrier proteins was performed, the reduced levels in the plasma and liver suggest reduced synthesis. Insulin administration normalized plasma and hepatic vitamin A concentrations. The duration of the present studies was short, only 4-6 weeks; therefore care must be taken in directly extrapolating these results to humans situations. However, changes observed here are important considering that human diabetes is a life long disease and is associated with later complications which may be aggravated further by vitamin A deficiency.

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